Cell and tissue culture of tomato: application to disease resistance to powdery mildew (Erysiphe cichoracearum) and leaf spot (Xanthomonas campestris pv. vesicatoria)

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Cell and Tissue Culture of Tomato: Application to Disease Resistance to Powdery Mildew (*Erysiphe cichoracearum*) and Leaf Spot (*Xanthomonas campestris pv. vesicatoria*)

Submitted by Mohamed A. Ali for the degree of Ph. D. of the University of Bath

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Dedication

TO THE MEMORY OF MY MOTHER

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Abstract

Somaclones were regenerated from hypocotyl, leaf, cotyledon and stem internode explants of tomato cvs Moneymaker and Rootstock with an eventual aim of generating lines with disease resistant to two major diseases of tomato. The effect of tomato genotype, explant type, and the combination of plant growth substances on the morphogenetic responses was established. Shoot regeneration from explants of tomato cultivar Moneymaker was induced by cytokinins (BAP) alone, but on explants of cv. Rootstock shoot regeneration was induced by BAP as well as combinations of BAP with either NAA or 2,4-D. The number of shoots produced at any concentration within the optimum concentration were not significantly different between the explants of the same genotype. No shoots were produced from callus. Tissue culture induced genetic and phenotypic variations in the plants regenerated from both cultivars.

Interaction of *Xanthomonas campestris* pv. *vesicatoria* was investigated *in vivo* with susceptible and resistant genotypes of pepper and tomato, and *in vitro* with cells of tomato cv. Rootstock (susceptible) in suspension cultures. Populations of pathogenic strains of *Xcv* increased in the susceptible pepper cv. Earlycalwonder and tomato cv. Rootstock, while the population growth of a non-pathogenic mutant was limited and it failed to induce symptoms on the susceptible genotypes of tomato or pepper. The rate of population growth of pathogenic strains was limited in tissues of the resistant tomato cv. Hawaii 7998 in which a hypersensitive reaction was induced at higher inoculum concentration (10^{8} cfu ml^{-1}).

Cell killing of tomato cells in suspension cultures by the pathogenic strains of *Xcv* and by the non-pathogenic mutant was investigated. Significant differences were found between the cell killing ability of the pathogenic and non-pathogenic bacterial strains and this was affected by the different growth phases of the cell

suspension cultures. Thus cells in exponential phase were more resistant than those in lag and stationary phases. The population of all bacterial strains increased 200-300 fold in the bioassay with tomato cells. Dialysable bacterial toxin(s) were not responsible for the cell killing.

Temperature and humidity relations of tomato powdery mildew *Erysiphe cichoracearum* were investigated in addition to the primary infection on susceptible tomato cv. Moneymaker. The conidia of *E. cichoracearum* germinated at wide ranges of temperature (15-35 °C) and humidity (5-100%). The optimum temperature for the germination and germ tube elongation were 20 and 25 °C respectively and the optimum humidity was 100%. The implication of these temperature and humidity relations on the ecological distribution of two species of tomato powdery mildew are discussed. Penetration was directly through the cuticle from lobate appressoria. The wild tomato accessions LA458 and LA2747 were resistant to powdery mildew. Conidial germination was significantly reduced on leaf discs of these accessions compared to cv. Moneymaker. Water and chloroform washings from leaves of Moneymaker and from LA2747 and LA458 significantly reduced conidial germination; germination on water and chloroform washings from LA2747 and LA458 was reduced significantly compared to Moneymaker. The germination on the chloroform washings from both lines were about half those on the water washings. The infrequent penetrations that occurred on the resistant lines gave rise to a hypersensitive response.

The tomato somaclones showed significant differences in the number of *E. cichoracearum* colonies per plant, however, no resistant line was identified. Differences between the somaclones in their interactions with *Xcv* strain E3 were also found. The potential use of somaclonal variation for obtaining disease resistance was demonstrated in this study.

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Introduction

Tomatoes, which are grown both in home gardens and commercially, are one of the world's most popular vegetables (Villareal, 1980). They are a good source of vitamin A and C, and can help alleviate deficiencies in these vitamins in the diet of people in many developing countries. Because tomato growing is labour intensive, tomatoes are an attractive cash crop for small farmers and potential sources of rural employment.

Powdery mildews can be one of the most destructive parasitic fungi of tomato (Omer, 1983; Correll, 1986). Their wide host range and ability of conidia to germinate at low relative humidity enable the disease to occur in many countries (Omer, 1983). Epidemic outbreaks of the disease can inflict enormous losses on tomato with yield reductions up to 40% being reported by Jones and Thomson (1987). Although resistant tomato cultivars do not exist, differences in the susceptibility of varieties, breeding lines and wild species have been reported by several authors (Palti, 1970, 1971; Kontaxis and Van Marnel, 1978; Correll, 1986 and Moens et al., 1985). In the absence of resistance genes in commercial cultivars, fungicide applications have been successfully used in controlling the disease (Omer, 1983; Correll et al., 1988; Jones and Thomson, 1987; Francheschiimi et al., 1983). However, the early stages of the disease infection may not be noticeable by most farmers (Reuveni and Rotem, 1972), and the general move towards reducing the use of chemicals in agriculture necessitates a breeding programme for the development of a cultivar(s) resistant to powdery mildews.

Tomato leaf spot, caused by *Xanthomonas campestris* pv. *vesicatoria* is another important disease in the tropics (Pohronezny and Volin, 1983). Under conditions of high temperature and frequent rains, yield losses can be 52% of marketable fruit weight as estimated by Pohronezny and Volin (1983). Chemical

control during periods of high disease pressure may be inadequate (Conover et al., 1981) and the pathogen develops resistance to the bactericides. (copper compounds) in a short period. One tomato cultivar, Hawaii 7998, is reported to have foliar resistance to the disease (Scott and Jones, 1986), which is controlled by quantitative gene action with small or negligible dominance. This remains to be transferred to the popular tomato cultivars using a modified backcrossing scheme(s) (Scott and Jones, 1989).

Both diseases (powdery mildew and leaf spot of tomato) affect the yield and quality of tomatoes produced. Sources of resistance to the diseases appear to be very limited. Thus conventional approaches of plant breeding for disease resistance need to be supplemented by alternative strategies. Plant cell and tissue culture techniques have a major role in crop improvement including selection for and generation of disease resistance. In addition these techniques offer considerable scope for enlarging the pool of genetic variation for increasing selection efficiency in imparting advantages of yield, stress resistance (both biotic and abiotic), improving quality to crop plants for mass propagation of chosen genotypes and for directing the process of genetic change (Ingram and Helgeson, 1980).

Project Aims

The aims of this project were as follows:

(1) To develop tissue culture techniques to produce somaclones from several tomato cultivars which could be screened for resistance to powdery mildew and *Xanthomonas campestris* pv. *vesicatoria*. The objective of this part of the research programme was to fully investigate the use of plant growth substance and explant types to obtain adventitious shoot regeneration from disorganised callus tissue. Further, it was intended to quantify the extent of somaclonal variation, for example through the measurement of ploidy level, plant growth and development.

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(2) To investigate the temperature and humidity requirements of powdery mildew in order to determine the optimum conditions for the disease maintenance and screening tomato somaclones for resistance to disease. It was intended to broaden this approach to consider the infection mechanisms of powdery mildew on susceptible tomato varieties, and any resistant somaclones, species or varieties available.

(3) To investigate the interaction between *Xanthomonas campestris* pv. *vesicatoria* and different genotypes (susceptible and resistant) of tomato and pepper *in vivo*. The first stage would be to investigate symptoms development, bacterial growth *in planta* and the consequent cell damage caused by different inoculum concentrations of the pathogenic and non-pathogenic strains. The second stage would be to investigate the interaction between *Xanthomonas campestris* pv. *vesicatoria* and susceptible tomato cells in suspension. The objective of this part was to assess the cell killing ability of different strains of the pathogen with tomato cells from different growth phases and the possibility of toxin production; any toxin found could possibly be used as an agent for selection of resistant varieties. Somaclones produced through tissue culture should also be screened for resistance to *Xanthomonas campestris* pv. *vesicatoria*.

Chapter (1)

Literature Review

1.1 Tomato

1.1.1 Origin, Distribution and Taxonomy

The commercial tomato *Lycopersicon esculentum* Miller evolved in the Andean region of South America, the natural habitat of eight wild species (Rick, 1976). Rick and Fobes (1975) suggested that the evolution of modern tomato cultivars followed from a hypothetical phylogeny precursor to *L. esculentum* var. cerasiforme, through domestication in Mexico to the present large-fruited cultivars. Shortly after the Spanish conquest, *L. esculentum* was carried to Europe in the sixteenth century and on to North America in the eighteenth century (Rick, 1976).

The commercial tomato (*L. esculentum* Mill.) is a member of the genus *Lycopersicon*, within the large family *Solanaceae*, consisting of nine species all of which have 12 pairs of chromosomes. Crossing of tomato with the other *Lycopersicon* species is either difficult or impractical and accordingly the species are divided into two complexes: Those which can easily be crossed with the commercial tomato are called the *Esculentum*-complex, and consists of (*L. esculentum* Mill., *L. pimpinellifolium* (Jusl) Mill., *L. cheesmanii*, *L. parviflorum*, *L. chmielewskii*, *L. pennelii* and *L. hirsutum* Humb. and Borpl.). The species which have genetic barriers to hybridization are categorised as the *peruvianum*-complex, which includes *L. chilense* Dun. and *L. peruvianum* (L.) Mill., both of which are self incompatible and unilateraly incompatible with *L. esculentum* while in compatible combination the embryos abort (Hogenboom, 1972 a-d).

1.1.2 Economic Importance of Tomato

Although tomato was cultivated as early as the 16th century, it was grown only as an ornamental plant for colour of its fruit, as the fruit was considered to be toxic due to the alkaloid tomatine. The cultivated tomato has a low concentration of this alkaloid compared to other species of *Lycopersicon* (Juvik et al., 1982). A positive correlation between the mortality of the larvae of the tomato fruit worm *Heliothis zea* with fruit tomatine content was established by Juvik and Stevens (1982) indicating its antibiotic activity. In addition Juvik and Stevens (1982) found that the larval growth rates and adult weights of *H. zea* were negatively correlated with the toxin content. In the second half of the nineteenth century, *Lycopersicon esculentum* became generally recognized as a highly valuable and nutritious food crop. Tomato is one of the major vegetable crops and is grown throughout the world with an annual production of 70 million metric tons (mt). During the 8-year period 1981 to 1989 the world production has increased by 31.8% from 51840 x 10^3 mt to 68328 x 10^3 mt (Table 1.1). Table 1.2 lists the ten largest tomato producing countries world-wide (FAO Year book 1989). The United States, the top world producer, also imports a significant quantity of tomatoes (Table 1.3). Several countries that are large producers (e.g. United states, Spain and Romania) also export a large quantity of tomatoes. On the other hand, some countries (e.g. Netherlands, Mexico and Jordan) produce largely for export. The area of tomato production in the developed countries, primarily in North America and Western Europe, has increased by 4.6% in this period compared to a 23.4% increase in the area of production in developing countries. This increase was highest in the Near East (28%) and Africa (38.4%). The yield in developed countries was nearly twice (1.8) that of the developing countries. This is due to the favourable conditions to tomato production and availability of technical information and finance to the growers in the developed countries. In the developing countries however, the climate is not always suitable

Table 1.1: World tomato production in 1989 based on FAO yearbook 1989

Countries	Area harvested (1000 ha)		Yield (kg/ha)		Production (1000 mt)	
	1979/81	1989	1979/81	1989	1979/81	1989
Dev.ped countries	544	569	37479	47158	20390	26835
N. America	176	202	42079	53516	7427	10803
W. Europe	322	321	34568	43532	11121	13974
Oceania	10	10	27099	36851	265	363
Oth. developed	36	36	43806	47083	1577	1695
Dev.ping countries	1009	1245	16409	1861	16555	24734
Africa	169	234	11064	11262	1872	2632
Latin America	276	312	18489	22314	5092	6959
Near East	415	531	20129	25876	8365	13752
Far East	148	168	8253	8259	1222	1386
Oth. developing	1	1	9558	9315	5	6
Asian	292	348	14243	15799	4157	5498
E. Europe + USSR	559	561	19198	20088	10738	11261
Developed all	1103	1129	28211	33734	31128	38096
Developing all	1301	1593	15923	18974	20712	30232
World	2404	2723	21565	25096	51840	68328

Table 1.2: Leading countries for world production of tomatoes based on FAO yearbook 1989

Country	Production Metric tons (1000)
U.S.A	10255
USSR	7100
Italy	5756
Turkey	5650
China	5430
Egypt	4800
Spain	2876
Brazil	2387
Romania	2200
Greece	2180

Table 1.3: Tomato import and export based on FAO yearbook 1989

Export		Import	
Country	Metric tons	Country	Metric tons
Netherlands	571355	Germany	455119
Mexico	439132	USA	393703
Spain	40425	France	329006
Jordan	193116	U.K.	272579
Bel-lux	141459	Canada	145496
Morocco	110000	Saudi Arabia	130000
Romania	90000		
USA	81752		
Bulgaria	80000		
Turkey	78446		

and the production techniques are not well developed. The tomato is grown both in open cultivation and in greenhouses for sale and home consumption. Greenhouse production is concentrated in western Europe to ensure year-round availability for fresh market consumption. Tomato use is broadly categorized as either fresh market or processed (i.e. canned tomatoes, juice, catsup, chilli sauce and paste). Tomatoes can increase the income of farmers and generate rural and urban employment and expand exports.

1.1.3 Breeding Accomplishments and Goals

Efforts for tomato improvement increased according to its popularity. Most of the effort has been directed to the yield and adaptability. Many tomato cultivars have been developed to suit cultivation under different systems and climates. For example cultivars suitable for greenhouse cultivation generally differ from their outdoor counterparts because of the vastly different cultural systems used in production. Many tomato breeding objectives encountered today involve fitting the crop to its intended environment, cultural system, method of harvest and/or handling and proposed food use.

Improvement of fruit quality is one of the objectives of most breeding programmes. Attempts have been made by Stevens and Rick (1986) to increase fruit solids contents and to alter the fruit acid content. These efforts have not been successful because of the complex interaction between the various components of tomato (Stevens, 1979, Villareal, 1980). The negative relationship between yield and solid contents (Stevens and Rick, 1986; Stevens, 1979, Villareal, 1980) is one of these complexities.

Improved yield and quality are universal goals of most breeding programmes; however the individual components that contribute to yield or quality, such as disease resistance, earliness and growth habit are equally important. Several

simply inherited genetic characters have had important impacts on the improvement of new cultivars and the changes in culture made possible by their development. One such gene is self-pruning (sp), (Rick and Butler, 1956). This results in compact growth, fewer nodes between inflorescences, early and more concentrated flowering, a feature essential for machine-harvestable cultivars. The determinate growth habit made possible the use of higher plant populations to increase yield per unit area and increase the number of harvests.

Numerous simply inherited characteristics are part of the genome of modern-day tomato cultivars (Stevens and Rick, 1986). Single genes also control resistance to many of the common diseases and dominance of resistance has facilitated the development of hybrids with resistance to as many as eight different pathogens (Gilbert et al., 1961). Much of this disease resistances originated in the wild relatives of the tomato and transferred to adopted cultivars of *L. esculentum* (Rick, 1982). More efforts will continue to overcome the existing breeding barriers between these wild species and the commercial tomato by mutation or embryo culture to enable the incorporation of useful traits into the popular tomato cultivars (Hogenboom, 1972 a-d).

As the tomato is expanded into new geographical regions, new pests may become economically important. *Phytophthora infestans* has become an important pest in Brazil (Maschio and Sampaio. 1982), but it is absent from most locations in the USA. Also new strains of existing pathogens will continue to appear. A third race of *Fusarium* wilt has been reported in Australia (Grattidge, 1982). As nutrients are depleted from arable land and interest accumulates in using poorer lands, breeding for stress has attracted much attention. Wild species germplasm contain a unique source of stress tolerant characteristics including salt tolerance in *L. cheesmanii*, drought tolerance in *L. hirsutum* and *Solanum Lycopersiciodes*, heat tolerance in *L. pimpinellifolium* and moisture tolerance in *L. esculentum* cv. cerasiforme (Rick, 1982). However, no new stress tolerant commercial cultivars

have yet been developed using these wild species. The complexity of inheritance, differential interactions at various stages in the life cycle and lack of efficient screening procedures or economic incentives are obstacles that have slowed progress (Rick, 1982, 1987). However, efforts are currently being directed towards developing new stress tolerant varieties using these wild species (e.g. Epstein et al., 1980). Other techniques such as plant tissue culture have been used to investigate water stress tolerance of tomato cells (Hasegawa et al., 1984) and selection of cells resistant to heavy metals (Stavarek and Rains, 1984). Trolinder and Shang (1991) recovered *in vitro* variants of cotton *Gossypium hirsutum* resistant to high temperature stress.

The present production of hybrid varieties of tomato using hand emasculation is tedious and costly and it has been suggested that introduction of the useful male sterile system would result in several new inexpensive hybrid varieties (Rick, 1982). Future research on the tomato will work towards such a male sterile.

1.1.4 Conventional Breeding for Disease Resistance

Numerous diseases adversely affect the growth, productivity and quality of the tomato (Yang, 1979). Ideally, disease control requires preventative measures and host plant resistance. The use of a disease resistant variety is the most effective and inexpensive means of control (Russell, 1978). Host resistance has been particularly important for the control of soil-borne pathogens such as *Verticillium* and *Fusarium* wilts, for which chemical control has been relatively ineffective and costly (Russell, 1978). As with other crops, a major breeding effort with tomato has been directed towards improved disease resistance. Disease resistance has been exploited to a greater extent than any other characteristic. Resistance to 30 different diseases of cultivated tomatoes has been detected in wild species and resistance to 16 of these has been bred into commercial cultivars (see Nevins, 1987). Resistance to several pathogens has been combined into true

breeding cultivars and resistance to 10 incorporated into F1 hybrid cultivars (see Nevins,1987). The hybrid cultivar Floramerica for example, possesses monogenic resistance against 5 diseases and tolerance of five others; additionally, it is free of three physiological disorders (Crill et al., 1972, Rick, 1982).

Resistance to several bacterial, fungal and viral diseases has been introduced from wild *Lycopersicon* species into cultivated tomato (Rick, 1982). These include *Fusarium* and *Verticillium* wilts, late blight and Tobacco Mosaic Virus. In most cases, such as *Cladosporium fulvum*, resistance is controlled by a single dominant gene (Kerr et al., 1980), often against a surprisingly broad spectrum of pathogenic races (Barksdale, 1972). *L. peruvianum* and *L. pimpinellifolium* have been a particularly useful source of resistance to rootrot nematodes (Stevens and Rick, 1986). Because of its close relationship with modern cultivars and ease of backcrossing, *L. pimpinellifolium* has frequently provided an attractive source of germplasm for plant breeders (Rick and Stevens, 1986).

1.2 Somaclonal Variation

The term "somaclone" and "somaclonal variation" were used by Larkin and Scowcroft (1981) to describe "plants regenerated from any form of cell culture" and the "variation amongst such plants".

1.2.1 Factors Influencing Somaclonal Variation

Generally, the variability detected in regenerated plants is due to either preexisting genetic variability in the tissues of the original explants or induced by some aspects of the tissue culture procedure. D'Amato (1978) has stated that based on nuclear genetic differences between differentiated cells and genetic phenomena occurring in the first few mitotic divisions of callus formation, most

explants comprise a heterogeneous cell population. For the understanding of somaclonal variation the influence of genotypes, explant source, duration of culture and culture conditions will be discussed.

1.2.1.1 Genotype

The genotype of plants used for *in vitro* regeneration of plants is an important variable because of its possible effect on the frequency of regeneration and the frequency of somaclones. The effect of the tomato genotype and the ploidy level of the plant explant on the stability of the ploidy level of regenerants was investigated by Koornneef et al. (1989). They regenerated tomato plants from leaf explants, calli and protoplasts of haploid and diploid tomato plants. Haploids were unstable and the number of regenerants that retained the original ploidy level differed significantly between the tested haploids. Seeni and Gnanam (1981) induced plant regeneration on calli derived from different tomato genotypes (homozygous (xa-2/xa-2) green; heterozygous (Xa-2/xa-2) yellow-green; and homozygous (Xa-2/Xa-2) albino). A high percentage (10-15%) of the tomato regenerants obtained from cultures of heterozygous individuals were of the varigated type. However, homozygous lines (green and albino) regenerated homozygous plants.

The frequency of organogenesis of calli of 4 tomato genotypes was investigated by Garcia-Reina and Luque (1988). Organogenesis of the calli and the number of plantlets regenerated per callus varied between the genotypes. In addition the genotype (Salvaje) which had higher number of calli with organogetic developments, also produced the highest number of shoots per explant. Genotypic differences in the morphogenetic ability of tomato and the number of regenerated shoots were also reported by Ohki et al. (1978), Tal et al. (1977), Di Paola et al. (1983) and Kurtz and Lineberger (1983). The shoot-forming capacity from leaf explants of 21 tomato genotypes and the heritability of this character were

evaluated (Frankenberger et al., 1981a). The shoot-forming capacity was variable between the different genotypes producing 2.9 and 14.6 shoots per explant of the genotypes Heinz 1350 and Redblush respectively. Diallel investigation of this character proved that it was highly heritable and controlled by recessive genes in 2 out of the 3 high shoot-forming genotypes.

1.2.1.2 Explant Type

Tetraploid tomato plants were regenerated from several types of explant from different genotypes of tomato, e.g. tetraploids were obtained from anther and stem internode cultures of *Lycopersicon peruvianum* (Sree Ramulu et al., 1976) and after protoplast culture of *L. pennellii* (Tan et al. 1987) and *L. esculentum* (Benton et al., 1986). Tetraploids were also observed among plants regenerated from leaf explants of *L. esculentum* (Evans and Sharp, 1983), although the authors did not report the frequency. Sibi (1982, 1986) tested 70 plants which were regenerated from callus cultures of tomato cv. Monalbo and found variable chromosome numbers, without specifying their numbers. In addition 5 plants with excessive branching had either a diploid or a variable number of chromosomes. Koorneef et al., (1989) investigated the effect of explant source on the stability of the ploidy level of plants regenerated from cell and tissue cultures of tomato. They found that regenerants from leaf explants of diploids were predominantly diploid in contrast to the plants regenerated from established callus cultures and protoplasts where the majority were tetraploid. The same results were found on regenerants from mesophyll protoplasts or leaf slices of an F_1 tomato hybrid between *L. esculentum* and *L. pennellii* (O'Connell et al., 1986). The effect of the explant source on somaclonal variation of tomato regenerants was also reported on the cultivar Moneymaker by Van den Bulk et al. (1990). Using leaf, cotyledon and hypocotyl explants, various morphological changes were recovered after plant regeneration. These were not stable except for ploidy variation which

was transmitted to the progenies of the regenerants. Variation in ploidy was mainly tetraploid and the frequency of polyploid plants depended on the explant source. Among 200 plants regenerated from hypocotyl explants 58% were polyploids, but only 1.5 and 11.9% polyploid plants were found when leaf and cotyledon explants were used respectively. The flow cytometric measurement of the nuclear DNA content of the 3 explants of the same age as the ones used for regeneration suggested that hypocotyl explants had a mixture of diploid and polyploid cells at a higher percentage compared to the leaf and cotyledon explants (Van den Bulk et al., 1990). These results indicated a correlation between the percentage of polyploid cells present in the explant material *in vivo* and the frequency of polyploid plants. Correspondingly the shoot-forming capacity of 8 tomato genotypes was found to be higher from cotyledon explants than from hypocotyl explants (Frankenberger et al., 1981b). Garcia-Reina and Luque (1988) found that the morphogenetic capacity of cotyledon, shoot apex and hypocotyl explants of 4 tomato genotypes were different when cultured on the same medium, but the cotyledonous explants had the highest morphogenetic frequency in all genotypes.

1.2.1.3 Culture Duration

Prolonged periods of tissue culture have frequently been reported to result in an increased frequency of genetic instability. Nayak and Sen (1987) investigated the relative cytological stability of callus and regenerated plants of *Ornithogalum thyrsoidas*. They noted that the frequency of diploid cells decreased with culture duration from 87% to 59% after 300 days in culture, whilst the tetraploid cells in the same callus culture increased from 13% to 41% during the same duration. However, no high level of ploidy other than tetraploid was found either in the original explant or old and new callus. Sree Ramulu et al. (1986) studied variability in the DNA level of calli produced from different potato

genotypes (haploid, diploid, and tetraploid) after one and two weeks of culture. The DNA level increased with time in all calli. The chromosome numbers of cells from these calli showed that polyploid and aneuploid cells occurred after 7 days in culture and increased in proportion with the elongation of the culture period. Organogenesis of calli of different tomato genotypes was reduced by the length of time in culture on callus-inducing medium (Di Paola et al., 1983). In oats the frequency of cytogenetically abnormal plants (such as aneuploids, chromosome breakage, loss, and interchange) increased dramatically with increased time in culture (McCoy et al., 1982). In contrast, Van den Bulk et al. (1990) did not find any effect of duration of tissue culture period (5 and 11 weeks) on the mutation frequency of plants regenerated from leaf explants of tomato cv. Moneymaker. Normal and genetically stable tomato plants were also regenerated from 2-year old callus produced from explants of chlormequat pre-treated tomato cvs Canatella and King Plus (De Langhe and De Bruijne, 1976).

1.2.1.4 Culture Conditions

Somaclonal variation has been induced in *in vitro* regenerated plants from tomato (Van den Bulk et al., 1990), wheat, cotton and Brassica (Bajaj et al., 1986). This was demonstrated for tomato by Evans and Sharp (1983) who regenerated somaclones from leaf explants of tomato cv. UC82B and identified 13 nuclear gene mutations among 230 progenies of these regenerants. Each mutant was from a separate explant and constituted a separate mutagenic event. In contrast, no variant was observed among 2000 plants from the seeds of the original cultivar. The mutagenic effect of plant growth substances on the regenerants, either singly or combined remains to be proved (see Evans 1986), but higher concentrations of BAP (5 and 10 μM) was reported to induce variability on regenerants of an ornamental *Nicotiana*. Also 2,4-D has been associated with the increased variability among *Hordeum* regenerants (Deambrogio and Dale,

1980) and potato (Shepard et al., 1980), but in both reports the effect of 2,4-D was associated with longer duration of culture. It is difficult to ascribe the increase in variability between the regenerants to the increase in the concentration of plant growth substance alone.

1.2.2 Origin of Tissue Culture Instability and Variability of Somaclones

Plants regenerated via tissue culture have shown changes in different characters compared to the original source of explants as discussed previously. Some of these changes are controlled by genetic factors and can be transferred to subsequent generations, but others are not expressed in the sexual progenies of the regenerants. Understanding of the mechanisms involved in producing the variation among the regenerated plants would help in the control of this process according to the reseach objectives.

1.2.2.1 Genetic Basis

Plants regenerated through tissue culture which have a variable chromosome numbers, mainly tetraploidy, have been reported in tomato (Van den Bulk et al., 1990). These polyploid plants had reduced fertility. Chromosome numerical variations were also reported on regenerants from protoplasts and plant tissues of potato (*Solanum tuberosum*), wheat (*Triticum aestivum*) (Karp, 1986) and different cereal crops (Vasil, 1986; Lorz et al., 1986; Maddok, 1986). Vasil (1986) investigated the genetic stability of regenerated plants from gramineous species and the nature of cell culture used for plant regeneration. It was concluded that variability was higher in plants regenerated from multicellular origin, which is the case in the vast majority of angiosperms. However, in contrast the majority of embryonic cultures of the gramineae retained their ploidy levels during *in vitro* growth (Vasil, 1986). However, Sreenivasan et al. (1987) reported extensive somaclonal variations on plants regenerated from the callus of one cultivar of

sugarcane Co 7704 due to aneuploid chromosome variation, whereas a limited morphological variability was shown by the regenerants of the other cultivar Coc 671. These results suggested that chromosome numerical variation is not a pre-requisite for somaclonal variation, but enhances the frequency of extreme variants.

Single gene mutations were also reported on plants regenerated from a diploid inbred cultivar of tomato (Evans and Sharp, 1983). These mutations (13 dominant and recessive genes) were transferred to the progenies of the tomato regenerants in a 3:1 ratio. Other genetic changes that happened *in vitro*, such as cytoplasmic gene changes, chromosome rearrangement, mitotic cross over and gene expression were discussed by Evans (1989), Larkin and Scowcroft (1981), Evans and Sharp (1986) and Evans et al. (1984). Karyotypic (gross) and cryptic changes associated with chromosome rearrangements, somatic gene rearrangements and gene amplification (increase in the copies of a gene per haploid genome) and depletion were also responsible for the variability among plants regenerated from an interspecific hybrid of *Nicotiana* (Venkateswarlu et al., 1987).

1.2.2.2 Molecular Changes

Variation in number of chromosomes has been reported by many authors Van Den Bulk (1990), Sree Ramulu et al. (1986), Huang and Chen (988), but somaclonal variation is not simply a reflection of chromosomal changes. More subtle changes in genomic DNA, or in the chloroplast and mitochondrial genomes are more difficult to detect. A marked reduction in the ribosomal rDNA unit of one family of regenerated plants of *Triticale* was found (see Brown and Lorz, 1986). This reduction in rDNA was heritable and because the original explants did not possess this character, it was concluded that the variability was created by the passage through tissue culture. Other molecular changes which could be the causes of somaclonal variation such as changes of chloroplast DNA and

mitochonderial DNA were discussed by Brown and Lorz (1986). The effect of tissue culture on DNA methylation, which affects gene expression was investigated in maize by Brown and Lorz (1986). They analysed the DNA from the progenies of selfed plants regenerated from embryonic callus cultures of the maize line A 188 which were showing phenotypic variations such as dwarfing, albinism and leaf stripes. They reported that tissue culture induced many changes in gene methylation, for both normal and variant plants, though no correlation can be determined between the degree of methylation and phenotypic variation. The results of Brown and Lorz (1986) suggest that, the molecular basis of somaclonal variation is not yet understood. A stable variant in the enzyme alcohol dehydrogenase (ADH) was identified amongst 645 maize plants regenerated from the callus of immature embryo (Brettell et al., 1986). This plant was identified amongst 5 regenerants from a single callus, hence resulting from the *in vitro* culture system.

1.2.2.3 Epigenetic Mechanisms

Cassells (1984) concluded that most of the tissue culture derived variability can be avoided if more attention is given to the cloning method for each cultivar as well as the rooting method, culture vessels closure and the complex water potential. Three types of variation, genetic, epigenetic and non-genetic which can result from tissue culture were discussed by Cassells (1984). A detailed analysis of the inheritance of the variability created by tissue culture was completed on the regenerants of tomato cultivar Monalbo (Sibi, 1986). She found that 56% of the variant progenies were mutants for 1 or more than 5 characters such as number of chromosomes, extensive branching, number leaves, days to flowering, vigour, plant height and length of the third leaf. A second class was identified (17%) where the phenotype was not true to type, but propagated by selfing without any segregation of quantitative (e.g height, leaf size, growth parameters)

and qualitative characteristics (branching type) for two generations, thus behaving as a homozygous line. The progenies from the reciprocal crosses among the last group, and with the cultivar itself were asymmetric (markedly paternal) and showed a heterosis-like effect which was neither caused directly by heterozygous coding DNA, nor by cytoplasmic change alone. The results of Sibi (1986) suggested heritable changes in cytoplasmic or nuclear genetic elements other than coding genes, which she termed as "epigenic changes". Other forms of epigenetic changes caused by ethylene accumulation, methylation and molecular cytoplasmic changes are reviewed thoroughly in Karp (1991).

1.3 Somaclonal Variation and Selection of Disease Resistance at the whole Plant Level

The recovery of stable heritable traits controlled by single gene mutations (Evans and Sharp, 1983) makes tissue culture/somaclonal variation a unique technique which could have a major impact on plant improvement by creating novel variants. However, limitations to the use of somaclonal variation are imposed by the type and frequency of variation and by the unwanted variation which often arises. Moreover, linkage between desirable and undesirable changes may result. Selection of disease resistant mutants from tissue culture without the application of any selection pressure was reported on sugarcane *Saccharum officinarum* variety Pindar (Krishnamurthi and Tlaskal, 1974). Thirty eight sub-clones were regenerated from callus induced on apical meristem explants and tested for Fiji disease resistance. Four of the sub-clones proved to be highly resistant to the disease. Variation in chromosome number was also found in 2 of the sub-clones. Somaclonal variants of sugarcane resistant to smut disease *Ustilago scitaminea* were also reported by Sreenivasan et al. (1987). An unselected population of 158 somaclones of the variety Co 7704 were tested for smut resistance. Fifty five percent were free from the disease throughout the cropping

season. Out of 97 somaclones of another susceptible cultivar of sugarcane (CoC 671), 67% were resistant to the disease (Sreenivasan et al., 1987). Plants resistant to *Xanthomonas campestris* pv. *pruni* were regenerated from embryo callus of two peach (*Prunus persica*) cultivars, susceptible Sunhigh and a moderately resistant Radhaven (Hammerschlag, 1990). Daub (1986) tested somaclones of tobacco regenerated from protoplast for resistance tobacco mosaic virus (TMV), *Meloidogyne incognita*, and *Phytophthora parasitica* var. *nicotianae* (causal agent of tobacco black shank). She noted an increase in black-shank resistance in both field and greenhouse tests on the progenies of the somaclones but found no resistance to the other pathogens. Progenies of tobacco somaclones produced by the same method were also found to have an increased levels of resistance to black-shank and bacterial wilt *Pseudomonas solanacearum* (Daub and Jenns, 1989). Successful selection at the plant level for somaclonal variants with an increased disease resistance has been reported by many workers (see Van Den Bulk, 1991). Evans (1989) was able to select a tomato line (DNAP 17) resistant to *Fusarium* race 2. This line was the third generation (R_3) of tomato somaclones regenerated from the leaf explants of tomato cv. UC82B and morphologically similar to the original source of explants. The resistance resulted from a single gene, dominant mutation. Brettell et al. (1980b) found 31 toxin-resistant male fertile maize plants out of 60 due to spontaneous mutation in the cytoplasmic mitochondria on these plants which were maintained for one year in culture.

Some studies have not succeeded in selecting regenerants with an increased disease resistance. Gavazzi et al. (1987) were unable to identify increases in resistance to TMV, *Verticillium wilt* or *Meloidogyne incognita* in somaclones derived from two susceptible tomato cultivars. Van Den Bulk et al. (1991) screened 3000 plants, progenies from somaclones regenerated from different explants of a susceptible tomato cv. Moneymaker, for resistance to bacterial canker caused by *Clavibacter michiganense* subsp. *michiganense*. A few plants

few plants showed a delay in disease development, but without any practical value. Failures to select somaclones with increased resistance to diseases were reported in different plant species. For example somaclones of tobacco to TMV or *M. incognita* (Daub and Jenns, 1989), sugarcane to rust and smut (Carls Schnell, cited in Daub and Jenns, 1989), and apple to the cedar-apple rust (Joung et al., 1987). A higher percentage of resistant regenerants can be expected if a preselection *in vitro* is applied (Sacriston, 1982, 1985),but the screening of unselected somaclones is easier and does not require the identification of toxins, or the determination of the optimum conditions for host-pathogen interaction. However, the chances of finding the required character are low and a large number of somaclones need to be screened.

1.4 Application of Cell and Tissue Culture and *in vitro* Selection for Disease Resistance Breeding

The *in vitro* selection of disease resistant plants can be useful when the required character is not available in the gene pool of the specific species. Selection is normally performed by two methods. The first one is to select for resistance to toxins produced by the pathogens directly or via their culture filtrates. Secondly pathogens can also be used as selection agents when no toxins are produced by them. These two aspects of selection will be discussed in the following sections.

1.4.1 Phytotoxin and Culture Filtrates as Tools in Breeding and Selection of Disease Resistant Plants

(A) Toxins:

A common approach for development of disease-resistant germplasm has been the use of toxins produced by a pathogen to select novel resistant variants in culture. Plant pathogens may produce two categories of toxins, host-specific

and host non-specific toxins. Host-specific toxins show the same specificity on the host as the pathogens and for most there is a clear evidence of roles in disease development (Buiatti and Ingram, 1991). Therefore plants which show resistance to these toxins should have some resistance to the pathogens producing them. Brettell et al. (1980) regenerated maize plants that were resistant to the host-specific HMT-toxin, produced by *Helminthosporium maydis* race T. The original corn line was carrying Texas male sterile cytoplasm (Tms), which coincides with disease susceptibility. Plants with normal cytoplasm are not affected by the toxin. Plants regenerated from toxin-tolerant Tms culture were fully resistant to the pathogen. More significantly, the tolerance expressed by cell culture was correlated with tolerance of isolated mitochondria to HMT-toxin. Studies on the progenies of these variants showed that male fertility and toxin resistance were inseparable, appeared to be located on the mitochondrial genome and were inherited together maternally (Brettell et al., 1980). Toxin resistant, male sterile plants were also regenerated from cultures without the exposure to the toxin (Brettell et al., 1980). This suggested that spontaneous mutation of mitochondria for toxin-resistance was also occurring in 50% of the cultures. Other toxin-resistant plants which were also resistant to plant pathogens were reviewed by Buiatti and Ingram (1991).

Non-specific toxins are usually considered as virulence factors, because many are not involved in the primary interaction that determines compatibility, but can influence the development of the disease (Van Den Bulk, 1991). Consequently plants resistant to these toxins may not be completely resistant to the pathogen, but because these toxins may suppress active defence mechanisms of the plants, toxin-tolerant variants may show increased disease tolerance. A number of investigators have used nonspecific toxins in cell selection schemes. The first was Carlson (1973), who selected mutants from populations of haploid tobacco (*Nicotina tabacum*) cell cultures and protoplasts that were resistance to methionine

sulfoximine (MSO), an analogue of the non-specific amino acid derived toxin (tabtoxin) produced by the tobacco pathogen *Pseudomonas syringe* pv. *tabaci.* Toxin-resistant calli were regenerated into plants which were also resistant to the toxin. The toxin resistance was transmitted to the progenies, but no correlation was found between the resistance to the toxin and to the pathogen at the whole plant level, because bacterial multiplication was not inhibited in the toxin-resistant plants. Fusaric acid, a nonspecific toxin produced by *F. oxysporum* f. sp. *lycopersici*, was used successfully for the selection of tomato plants resistant to the pathogen from protoplasts of the susceptible cultivar UC-82 (Shahin and Spivey, 1986). The resistance to *F. oxysporum* was controlled by a single dominant gene (Shahin and Spivey, 1986). Tomato plants regenerated from non-challenged cells were also resistant to *F. oxysporum* which suggests that the tissue culture conditions induced spontaneous gene mutations (Brettell et al., 1980; Larkin and Scowcroft, 1983; Ling et al., 1985). Nonspecific toxins such as sirodesmin PL and deacetylsirodesmin PL produced by *Leptosphaeria maculans* a pathogen of oilseed rape (*Phoma lingam)* may have a nonspecific role in pathogenesis, but were used for the selection of disease resistant hybrids of *Brassica* species (see Buiatti and Ingram, 1991).

Tissue culture techniques might provide alternative methods for evaluating the degree of resistance in the host. Crude AT toxin is produced in the culture filtrate of *Alternaria alternata* f. sp. *tobacco*, the causal agent of tobacco brown spot disease. This toxin inhibited the growth of both resistant and susceptible tobacco cultivars when added to their cell-suspension cultures, but cell killing was much less in the resistant compared to the susceptible cultivar when the viability of cells was assessed by staining methods (Ishida and Kumashiro, 1988).

The success of selecting disease resistant plants by means of non-specific toxins depends on the involvement in disease development, but this does not necessarily guarantee its suitability as a selection agent for disease resistance. The

mode of action may be a key factor in determining its practical use. Nachmias et al. (1990) noted that suspension cells of susceptible potato were affected by the Vd toxin produced by *Verticillium dahliae*, whilst protoplasts remained unaffected. Furthermore, only the callus cells which were in direct contact with the toxin medium were affected. This toxin acts on K^+ and Na^+ ion transport system in plasma membranes, and it was suggested that the isotonic condition during the protoplast assay interfered with the effect of the toxin on the plasma membrane (Nachmias et al., 1990). This observation might be explained by the inability of the toxin to diffuse into the tissue. Following the failure to select cercosporin-resistant mutants in tobacco and sugar beet cultures Daub (1984) also stressed the importance of the mode of action in the selection of disease resistance. The nonspecific toxin cercosporin is produced by members of the genus *Cercospora* in large quantities when grown in light (Daub and Hangarter, 1983). This toxin generates a singlet oxygen and superoxide in the presence of light and causes the peroxidation of unsaturated fatty acids in the membrane lipids leading to increase in cell leakiness observed in plant tissue following cercosporin treatment (Daub, 1982). Cercosporin is not toxic to plant cells in the dark, which suggested that it has a role to play in the disease development enhanced by light. However, cells of disease susceptible and resistant plants were killed by the toxin (Daub, 1986), because the unsaturated fatty acids in these and indeed in most cells are susceptible to peroxidation. In cases where plants resistant to toxins were selected in culture successfully, the mechanism of resistance was linked with changes in enzymes, levels of enzyme substrates or other proteins (Carlson, 1973; Negrutiu et al., 1984). Daub (1984) reported that the resistance found in some species is directed against the pathogen itself not to a toxin. Also many pathogens may not depend on toxins for their pathogenicity. Hence the selection at the whole plant level might be the most suitable approach for resistance against this pathogen.

(B) Culture Filtrates:

Crude culture filtrates can be used as selective agents, especially when they exhibit phytotoxic activities, in the absence of any characterised toxin. Toyoda et al. (1989) regenerated tomato plants resistant to bacterial wilt caused by *Pseudomonas solanacearum* from filtrate-resistant calli of tomato cv. Fukuju No. 2. Selvapandiyan et al. (1987) also succeeded in selecting tobacco cell lines resistant to the culture filtrate of *Fusarium oxysporum*. Hammerschlag (1984) claimed that a toxic metabolite in the culture filtrate produced by *Xanthomonas campestris* pv. *pruni* is involved in bacterial spot development of peach. In a selection experiment, callus cultures of peach initiated from the leaf spot susceptible cv. Sunlight were exposed to the toxic culture metabolite for several selection cycles, with progressively higher concentrations of the filtrate in each cycle. Two out of four plants regenerated from the surviving calli were significantly more resistant to bacterial spot than the parental cv. Sunlight. In addition, one clone had significantly more resistance than the moderately resistant cv. Redhaven (Hammerschlag, 1988). Alfalfa (*Medicago sativa*) plants resistant to *Fusarium oxysporum* f. sp. *medicaginis* were recovered from calli resistant to the culture filtrate of this pathogen (Arcioni et al., 1987). There was a high correlation between the resistance to the culture filtrate and disease resistance. Culture filtrates have to be used with care, as shown by Vardi et al. (1986) who investigated the suitability of *Phytophthora citrophthora* culture filtrate as an *in vitro* selection tool for resistance against the pathogen. They suggested that the toxic effect produced by the culture filtrate at the cellular level may result from secondary metabolites, possibly auxin-like substances, which had no role in disease development. Such selection schemes could risk the production of variants resistant to compounds other than the initial toxin.

1.4.2 Selection *In Vitro* with Pathogens

The pathogen itself can be used as a selection agent when no toxin or toxic metabolite can be identified. However, in this approach the pathogen may overgrow the host cells and the culture medium rapidly, hampering any observations. In addition, several other factors may also affect the resistance in tissue culture, including temperature, the medium components and inoculum concentration (Helgeson and Haberlach, 1980). The expression of resistance at cellular level is a prerequisite for the use of pathogens in selection programmes (Helgeson and Haberlach, 1980).

The use of pathogens as selection agents is more suitable for the selection of virus resistant plants. Toyoda et al. (1989) selected virus-resistant tobacco shoots regenerated from callus induced from axillary buds of systemically infected plants of tobacco cv Bright Yellow. The somaclones had a normal chromosome number (2n = 48) and the resistance to tobacco mosaic virus was due to a dominant single gene mutation. However, Palit and Reddy (1987) regenerated plantlets resistant to *Pyricularia oryzae* from irradiated rice calli of a susceptible variety Tellahamsa using the pathogen as a selection agent. Healthy growing calli were inoculated with a suspension of the pathogen (10^5 spores ml^{-1}). Plantlets were regenerated from the healthy areas of the callus which were not covered by the growth of the pathogen and 8 plantlets out of 104 exhibited resistance to the pathogen. However, the genetics of resistance were not described. Plants regenerated from embryonic tissue of haploid *Brassica napus*, obtained after infection with resting spores of *Plasmodiophora brassica* showed no resistance to the disease (Sacristan and Hoftmann, 1979).

1.4.3 Study of Host-Pathogen Interactions *In Vitro*

In vitro culture can facilitate study of host-parasite interactions because inoculation is synchronous, the components of media are known, and environmental conditions can be strictly controlled. Also the possibility of testing cell or tissues at different levels of differentiation make this system unique in the determination of host-pathogen interaction.

Witsenboer et al. (1988) tested the possibility of using the host-specific toxins (AAL-toxins) produced by *Alternaria alternata* f. sp *lycopersici* as selective agents for *in vitro* selection at the cellular level for disease resistance. Effects of AAL-toxins on leaves, leaf discs, roots, calli, suspension cells and protoplasts of susceptible and resistant tomato genotypes were investigated to gain insight into cellular effects of the toxin and into the mechanisms of plant insensitivity to AAL-toxins. Severe necrosis was observed only on the leaves of the susceptible genotype, whilst leaves of the resistant genotypes did not show necrosis. Inhibitory effects of toxins were observed at all other levels in resistant and susceptible genotypes: toxins inhibited shoot induction on leaf discs, root growth, and growth of calli, suspension cells and protoplasts.

In some cases the specificity of host-pathogen interactions in the intact host has been shown to occur at the cellular level, or by a group of cells using tissue culture and pathogen combination. Huang et al. (1988) reported that calli from wilt-resistant clones of *Solanum phureja* showed rapid browning followed by cell death when inoculated by strains of *Pseudomonas solanacearum* and the leaf showed typical HR. However, calli and leaves of susceptible clones retained their normal appearance for 48 h after inoculation. The death of callus cells was caused by a bacterial protein which was produced in abundance when the pathogen was in contact with plant tissues. This protein has a high proline and glycine content and causes rapid browning of callus cells, rapid death of suspension-culture cells, and a typical HR in the leaves of potato clone c-3. Miller et al. (1984) developed

a tissue culture system in which callus expressed the susceptibility and resistance of alfalfa *Medicago sativa* to *Phytophthora megasperma* f. sp. *medicaginis* (*Pmm*). Nonhost resistance to the soybean pathogen *P. megasperma* f. sp. *glycinea* (*Pmg*) was also expressed (Miller et al., 1985). Growth of *Pmm* was limited on the callus derived from resistant plants (M194), but the callus from susceptible plants M269 was completely covered by *Pmm* hyphae (Miller, 1984). Both resistant and nonhost calli appeared darker brown than the susceptible calli. Furthermore, the results of Miller et al. (1984) suggested that the concentration of kinetin in the growth medium was critical for both callus morphology and expression of disease resistance. Haberlach et al. (1978) also suggested the necessity of plant growth substances on the expression of disease resistance by tobacco *N. tabacum* L. against*Phytophthora parasitica* var. *nicotianae*. They reported that tissues from resistant cultivars exhibited an HR to zoospores of the fungus with 1.0 μM kinetin and 11.5 μM IAA or 1.0 μM 2,4-D and subsequently these tissues were colonized only slightly by the fungus, but the HR was not induced when susceptible cultivars or tissues from resistant cultivars were supplied with higher cytokinin levels (10 μM kinetin), in this case tissues were heavily colonized.

Atkinson et al. (1985) suggested that an HR can be induced in an incompatible interaction between host cells in suspension cultures and a pathogen. Net electrolyte efflux, decline in respiration rate, development of brown pigmentation, and finally cell death were observed in tobacco cells after inoculation with *Pseudomonas syringae* pv. *pisi* (incompatible with tobacco) or with the avirulent isolate BI of *P. solanacearum* strain K60. However, there was little electrolyte loss with bacteria which did not kill tobacco cells i.e. *Agrobacterium tumefaciens* (compatible with tobacco), *P. fluorescens* (a saprophyte), and heat-killed cells of *P. s. pisi*. Tissue culture provides an alternative means to investigate the formation of HR-eliciting signal in *P. syringae*. Some of the culture conditions which affect the initiation of the HR induced in the incompatible interaction between tobacco

cells in suspension cultures and *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *pisi* were investigated by Yucel et al. (1989). They suggested that the ionic response of tobacco cells was inhibited by the addition of rifampicin, tetracycline, and streptomycin during the 2-2.5 h induction stage. Co-culturing the bacteria with tobacco cells for 3 h before the inoculation of fresh tobacco cells was also found to abolish the sensitivity to these inhibitors and reduce the response of tobacco cells from 3 to 1 h. Yucel et al. (1989) suggested that the hypersensitive response-eliciting signal was formed late in the induction stage, perhaps as a result of the depression of some of the *P. syringae* genes functional in elicitation of the hypersensitive response.

In vitro techniques can be used for the identification of host-induced toxins which have not been detected by other approaches. Youle and Cooper (1987) reported toxic activity of filtrates from the interaction between apple cells and either a virulent or an avirulent capsulated isolate (T) of *Erwinia amylovora* when these filtrates were concentrated 35-fold. The size of this toxin is $\leq$ 1000 daltons.

Cell suspension cultures are useful for examining metabolic pathways and for obtaining rapid homogeneous contact between plant cells and a pathogen. Thus *Phaseolus vulgaris* cultures produce the phytoalexin, phaseollin, and many resistance related proteins when challenged with elicitors and fungi (Dixon, 1980). In addition soybean cultures challenged with a glucan elicitor isolated from the cell walls of *Phytophthora megasperma* f. sp. *glycinea* produced large qualities of phenylalanine ammonia-lyase and glyceollin (see Helgeson, 1983).

The interaction of host and pathogen is a complex process affected by many factors in both the plant and the pathogen. Plants have structures and mechanisms which may contribute to defense. These include leaf hairs, cell walls, cuticle, wax, hypersensitive response, cellular lignification, production of preformed and induced antimicrobial compounds. Pathogens, on the other hand may produce toxins, degradative enzymes, polysaccharides and other pathogenicity determinants.

At the cellular level some of these mechanisms may not operate due to the lack of some plant structure or receptor or a pathogen product may only be produced *in planta*. This problem has been well demonstrated with respect to the response of soybean callus culture or receptor to *P. megasperma* var. *sojae* where the extent of glyceollin production by cultures of different host cultivars was quantitatively different from that of the intact plant (Helgeson, 1983). Our general knowledge of the biochemical events that determine compatibility and incompatibility between plants and pathogens is poor for most diseases. Suspension cultures provide a potentially ideal system for studying aspects of these events and are employed in this study.

1.5 Tomato Bacterial Spot Disease Caused by *Xanthomonas campestris pv. vesicatoria* (*Xcv*) (Doidge) Dye

1.5.1 The Pathogen (*Xcv*) and its Economic Importance

Xanthomonas campestris pv. *vesicatoria* (*Xcv*) (Doidge) Dye, is a gram-negative bacterium which causes foliage and fruit spot disease of tomato (*Lycopersicum esculentum* Mill.) and pepper (*Capsicum annuum L.*). The disease is a serious yield-limiting threat in warm, humid areas of the world (Pohronezny and Volin, 1983). Foliar symptoms include the appearance of circular, water-soaked spots which becomes necrotic and measure from 1-5 mm in diameter. The tissue between lesions become chlorotic. Infected leaves turned brown, become dry and leathery, and drop (Volin, 1979; Nayudu and Walker, 1960). Although infection is not systemic in nature, a downward curling of the diseased foliage is a characteristic response to infection. All above-ground plant parts are susceptible, but the most serious losses are due to leaf infections and subsequent defoliation, which reduces yield and fruit grade due to cracking, sunscald, or

black shoulder (Nayudu and Walker, 1960). Reported losses under heavy disease severity have been estimated to be as high as 52% as measured by weight losses of marketable fruit (Pohronezny and Volin, 1983).

1.5.2 Factors Affecting Bacterial Spot of Tomato and Peppers

Relative humidity (rh) is a key factor that determines the development of many bacterial diseases in vegetables (Davis and Halmos, 1958; Yunis et al., 1980). High relative humidity (90%) in general and free water on leaf surface in particular (fog, irrigation water, dew) help the pathogen reach the infected site and enhance its multiplication at the set on of disease development (Panopoulous and Schroth, 1974; Rotem and Reichert, 1964). Furthermore, free water also favours secondary infection of healthy plants by most bacterial pathogens under field conditions (Diab et al., 1982). Frequent rains are conducive to development of this warm weather disease and complicate successful use of foliar sprays for control (Cook and Stall, 1982). Long periods at low rh irreversibly prevented the pathogen from initiating disease even if high rh was provided later (Diab et al., 1982).

The disease develops most rapidly at a continuous 24°C, the temperature favouring good growth of the host plant (Nayudu and Walker, 1960). They reported that a night temperature of 16°C suppressed the disease, regardless of day temperature. Older leaves, in the oldest plants, are less susceptible to infection (Nayudu and Walker, 1960; Davis and Halmos; 1958). Host nutrition was also found to influence disease development. Nayudu and Walker (1960) reported significant disease suppression at extremely high levels of nitrogen, phosphorous and potassium.

1.5.3 Survival Mechanisms of *Xcv*

Xcv, survives for long periods in tomato and pepper seeds (Jones et al., 1986; Crossan and Morehart, 1964). Infected seeds produce diseased seedlings under appropriate conditions, and diseased seedlings with or without symptoms can develop into diseased plants (Diab et al., 1982; Krupka and Crossan, 1956; Leben, 1962; Lewis and Brown, 1961; Peterson, 1963). Some *Xcv* isolates inhibit pepper seed germination but have no such effect on tomato seeds (Bashan, 1986). Weeds, included solanaceous weeds, have been shown to serve as reservoirs of bacterial pathogens (Jones et al., 1986). Crop residue and volunteer tomato plants have been very important for the survival of *Xcv* (Jones et al., 1986; Peterson, 1963). *Xcv* can be carried by a wide variety of agents including animals, man, insects, mites, agricultural tools, aircraft, soil particles, and water sources (Bashan, 1986). Of these, specific insects and tools commonly used for crop cultivation were the most heavily contaminated. Soil adhering to agricultural tools or carried by various water sources can also serve as a disseminating agent.

1.5.4 Genetics of Host-Pathogen Interaction

Strains of *Xcv* appear to interact with tomato and pepper genotypes in a gene-for-gene manner (Ellingboe, 1981). In a gene-for-gene interaction, a host is resistant to a particular pathogen if there exists in the pathogen an avirulence gene that corresponds to a dominant resistance gene in the host. Minsavage et al. (1990) distinguished three groups of *Xcv* on the basis of virulence on the tomato cultivar Walter and a set of near-isogenic lines of pepper. The near-isogenic lines of pepper were derived from Earlycalwonder (ECW), ECW-10R, ECW-20R and ECW-30R and contain the resistance genes Bs1, Bs2, and Bs3, respectively.

A partial description of the race structure of *Xcv* is described in Minsavage et al. (1990). The tomato group (*Xcv*T) is virulent on tomato only, the pepper

group (*Xcv*P) on pepper only, and the pepper-tomato group (*Xcv*PT) on both pepper and tomato. Within the *Xcv*PT and *Xcv*P groups, races of pathogen can be distinguished by their ability to cause disease on various pepper lines.

Three sources of resistance to strains of *Xcv* have been described in pepper (Cook and Stall, 1963; Cook and Guevara, 1984; Kim and Hartmann, 1985), and each resistance gene is specific for particular strains of the pathogen (Hibberd et al., 1987b). Furthermore, each resistance gene is simply inherited and each of the three genes segregates independently from each other (Hibberd et al., 1987b). Resistance in pepper to *Xcv* is generally associated with a hypersensitive response (HR). The HR is a local defence reaction accompanied by a rapid necrosis of the infected tissue (Klement and Goodman, 1967). However, the HR resistance of pepper to *Xcv*T is frequently overcome by pathogen change to pepper virulence (Dahlbeck and Stall, 1979), a characteristic that often indicates an interaction which is controlled in a simple genetic manner (Kearney et al., 1988).

Resistance to *Xcv* in tomato was not previously considered a practical option (Crill et al., 1972; Lawson and Summers, 1984a; Sotirovo and Beleva, 1975), because only horizontal resistance was available and the levels of resistance were not considered useful in a plant breeding programme. However, a new source of resistance, Hawaii 7998, was reported in 1986 (Scott and Jones, 1986). This genotype exhibited a hypersensitive response when infiltrated with strains of *Xcv* (Jones and Scott, 1986), as well as supporting lower bacterial populations in the lesions compared to susceptible tomato. Thus, a genotype with an adequate level of resistance that offered promise in breeding for bacterial spot resistance. The transfer of this resistance from Hawaii 7998 into horticulturally desirable genotypes has been difficult because of it's complex genetic control (Scott and Jones, 1989). This genetic control is largely additive with a small dominance component. Another tomato accession PI 248270 'Sugar' was observed to have a high level of resistance to fruit spot caused by *Xcv* despite having susceptible foliage in the

field (Scott et al., 1987). Hybrids between Sugar and Hawaii 7998 were similar in fruit spot (%) to 'Sugar', but had significantly less fruit spot (%) than Hawaii 7998, indicating a high degree of dominance for fruit spot resistance (Scott et al., 1987).

1.6 Powdery Mildews of Tomato

1.6.1 Biological Characteristics of Powdery Mildew and its Economic Effect on Tomato

The powdery mildew fungi (Erysiphaceae) are obligate pathogens which infect a wide host range. Hirata (1966) estimated that between 13-38% of Angiosperms in a given geographical region could be infected by them. Most of the vegetable crops used in temperate and tropical regions are included within this host range, many of which provide staple foods for populations in developing countries. In countries of the Mediterranean basin, powdery mildew of tomato is reportedly caused by *Leveillula taurica* (Reuveni and Rotem, 1973); only the oidial stage has been found in tropical countries (Correll et al., 1988; Omer, 1984; Kontaxis and Van Maren, 1978).

Another powdery mildew fungus, *Erysiphe cichoracearum* DC, occurs on tomato in several countries including U.K., France, Japan and Australia (Fletcher et al., 1988; Wicks and Clare, 1981). This pathogen is mainly associated with plants of the family Compositae.

Powdery mildew caused by *L. taurica* has resulted in 40% reduction in yield of tomato (Jones and Thomson, 1987). Also the amount of tomato fruit affected by the sun in inoculated plots was three to four times greater than in plots where the disease was controlled. However, Correll (1986) found no reduction in fresh fruit weight of susceptible tomato cultivars, caused by powdery mildew, when harvested at the " mature green" stage of development.

Tafradjiiski et al. (1975) have established that with tomatoes and eggplant,

L. taurica enters directly, by germ tube perforating the cuticle, but in pepper and leek, penetration takes place chiefly through the stomates. *E. cichoracearum* penetration ocurs directly through the cuticle (Staub et al., 1974). Both pathogens chiefly affect the leaves and are much less frequently encountered on other organs of certain hosts. The most common symptom consists of characteristic lesions covered with conidia on the upper leaf surfaces and occasionally on the lower surfaces of mature leaves within the crop canopy. Fruits and younger leaves are generally symptomless. In advanced cases of infection the spots coalesce and become necrotic and cover the whole of the leaf surface (Fletcher and Smewin, 1987; Kontaxis and Van Meren, 1987). Eventually the whole leaf blade may collapse and leaf wilting, desiccation and shedding can occur.

Infection by powdery mildew fungi involves the formation of a structurally complex host-parasite interface which results in the flow of host nutrients to the fungus without death of host cells. This biotrophic form of nutrition enables powdery mildew fungi to coexist for extensive periods with their hosts and to sporulate profusely (McKeen et al., 1966; Manners, 1989; Kunoh et al., 1979). Powdery mildew fungi exhibit a high degree of host species and cultivar specificity and race-cultivar specific interactions are frequent (Ellingboe 1972). Most powdery mildew fungi are epiparasitic i.e. all fungal structures except haustoria develop on the surfaces of host plants (Manners, 1989; Mckeen et al., 1966). Endoparastic powdery mildew fungi are *Phyllactinia* and *Leveillula*, which develop intercellular mycelium (Kunoh et al., 1979; Yarwood, 1957). The morphology of haustoria and related structures of *L. taurica* resemble, in many respects, those of *E. cichoracearum* (Kunoh et al., 1979; McKeen et al., 1966) and *Sphaerotheca pannosa* (Perera and Gay, 1976). Those of *E. cichoracearum* and *S. pannosa* are formed in epidermal cells, whereas those of *L. taurica* are formed in mesophyll cells. The haustorium is enclosed by an invagination of the host cell plasmalemma termed the extrahaustorial membrane and this membrane

is separated from the haustorial body by the extrahaustorial matrix. The mechanisms of transport of host metabolites to the haustorium and then translocation to the surface mycelium of powdery mildew have been partly elucidated (Manners, 1989).

1.6.2 Host Range and Geographical Distribution

According to Hirata (1968), the host range of *L. taurica* extends over 710 species in 290 genera and 59 families. Palti (1974) reported that at least 750 species including 27 major vegetable and field crops are hosts of *L. taurica*. The ability of *L. taurica* from one host species to infect other hosts belonging to different families, when artificially inoculated, has been demonstrated (Nour, 1958; Correll, 1986). The results of cross inoculation tests have in some instances been contradictory and have in any case been shown to be influenced by environmental factors (Nour, 1959).

Hirata (1968) has pointed out that alone among the genera of powdery mildew with large host ranges, *Leveillula* is restricted to fairly well defined climatic regions. It has been recorded in countries with warm climates in Asia, Mediterranean, Africa and the United States (Natour et al., 1971; Nour, 1958; Moens et al., 1985; Correll, 1986). Geographical distribution varies greatly with host species; it is widest in both dry and humid zones on hosts such as pepper but more strictly limited to dry conditions for other hosts (Palti, 1971). In contrast *Erysiphe sp.* is a common powdery mildew on tomato in Northern Europe countries (Fletcher, 1988), where *Leveillula* is rare or absent.

1.6.3 Epidemiological Considerations

Tomato and peppers are the most economically important hosts of powdery mildew *L. taurica.* However, many of the variables that affect powdery mildew of tomato are poorly understood, with epidemics often being sporadic and unpredictable. Palti (1971) pointed out that very little has been published on the epidemiology of *L. taurica* under field conditions.

Since sources of inoculum are usually plentiful, the actual outbreak of epidemics largely depends on interactions between the availability of susceptible host tissue and environmental factors. Powdery mildew was reported by Palti (1971, 1972) to be more important on mature tissue of tomatoes, but Correll et al. (1988) found that both immature and mature host tissue was susceptible to infection by *L. taurica* in the field, but there were considerably more lesions on older leaves than the younger ones within the same plant. They also found that onset of the disease was related to availability of inoculum and not to the physiological age of tomato nor to any specific metorological conditions. Hosts susceptible to powdery mildew were divided by Palti (1971) into two groups: in the first group infection depends clearly on weather conditions, and in the other group, no obvious dependence on weather could be demonstrated. Powdery mildew epidemics on tomato are dependent on low humidity according to Reuveni and Rotem (1972) and Palti (1971). The humidity level conducive to attack in this crop is related to the degree of varietal susceptibility. Thus moderately susceptible tomatoes are subject to mildew attack only in exceptionally dry summers, but highly susceptible varieties are also attacked in normal summers (Palti, 1971). When mildew attack is not clearly conditioned by weather, outbreaks of epidemics are predominantly linked with the stage at which the host plant reaches highest susceptibility. Since plants develop more rapidly under higher temperature, they will reach the susceptible stage sooner in warm weather. But the effect of temperature in this case is indirect (Palti, 1971).

1.6.4 Factors Influencing Disease Development

Of a large number of factors that influence disease development, four are the most important: moisture, temperature, light and soil fertility. It is not easy to distinguish the individual effects of the first three, and experiments designed to elucidate the effects of soil fertility have often given variable results. Comparative conidia germination tests on humidities have been carried out by Nour (1958) with conidia from various hosts and by Clerk and Ayesu-Offei (1967) and by Caesar and Clerk (1985) with conidia from *Capsicum annuum.* Correl (1986) also examined the germination of *L. taurica* conidia collected from tomato at different rh and temperature. Nour (1958) reported that conidia of *L. taurica* were able to germinate, on glass, under a wide range of humidities (0-100%). Germination was best at 85-100% rh with long germ tubes and turgid conidia, but was poor at lower humidities (0-30%) with short germ tubes and shrunken conidia. Infection by *L. taurica* was 30-40% more severe on furrow-irrigated compared to sprinkler-irrigated tomatoes (Rotem and Cohen, 1966) which suggests inhibition by free water. It has long been known that the internal structure of powdery mildew conidia collapses when the spores are in water, and immersion for 3 minutes can kill 50% of the conidia (Zaracovitis, 1966). Also free water can affect the contact and adhesion of conidia and germ tubes to the leaf surface and penetration of the leaf (Nour, 1958). The effects of humidity on conidia of *E. graminis* from barley gave similar results, except they are more sensitive to humidities below 95% rh as are the conidia of *E. cichoracearum, E. umbelliferarum* and *Sphaerotheca fuliginea* (Nour, 1958). Other workers have reported a similar effect of humidity on conidia of *L. taurica* from different hosts including pepper (Caesar and Clerk, 1985; Clerk and Ayesu-Offei, 1967) and tomato (Correll, 1986).

Yarwood et al. (1954) stated that for the powdery mildews as a group, optimum temperature for disease development is approximately 21 °C; this may

reflect several components of the pathogen such as germination, appressorial formation, and sporulation as well as influencing the host-pathogen interaction. Temperature always acts in conjunction with some combination of humidity and light, and the nature of this combination may determine the response to temperature. This is especially true with powdery mildews and other foliar fungal pathogens, where in nature high temperatures are usually associated with high light intensity and low humidity. They also reported that the variability in the optimum temperature for conidial germination within a species (e.g. *E. cichoracearum* or *E. graminis*) is about as great as between different species. *E. cichoracearum* from cantaloupe showed temperature optimum of 25 to 28 °C this same range has been reported as optimal for *E. cichoracearum* from tomato (Fletcher, 1988) which gave 94.6% conidial germination. Ward and Manners (1974) reported that sporulation of *E. graminis* was maximum at 20 °C, decreasing sharply at higher and lower temperature; the optimum rh was 100% and light intensity and photoperiod had little effect. The temperature and relative humidity effect was also investigated by Manners and Hossain (1963) on three strains of *E. graminis*. The optimum temperature for spore germination was 20°C and the optimum R.H was 100% for the three strains.

The higher incidence of mildew on shaded than on exposed leaves, seen particularly with tobacco (Hopkins, 1956), suggested that light is also an important factor, but this may in part be a temperature effects, since under tropical conditions there may be considerable differences in temperature between such leaves. There have been reports that conida collected in the afternoon gave a higher percentage germination than those collected in the morning (Hossain, 1959) or that the time of collection did not affect germination, but did affect the response the response of conidia to nutrients (Yarwood and Cohen, 1949). Ward and Manners (1974) suggested that the duration of the light period immediately before conidia were collected may be the factor controlling such diurnal effect.

Ultraviolet light is harmful to the germination of *E. graminis* conidia and their infectivity is even more sensitive (Moseman, 1966). The sporeling is less vulnerable to ultraviolet light after the haustoria have formed so that more radiation is required to kill colonies than to prevent infection. Nair and Ellingboe (1965) reported that the germination of conidia of *E. graminis* was insensitive to light intensities from 0-300 ft-c. The effects of soil fertility are equally complex as might be expected. Cole (1964) found that tobacco leaves were more susceptible to *E. cichoracearum* when grown in 50:38:50 ppm of NPK. In some instances increased susceptibility following various fertilizer treatments appeared to be linked with a growth response. Last's (1953) findings suggested that mildew severity is related to host vigour and that factors which favour plant growth correspondingly favour mildew development. On this theme Caesar and Clerk (1985) found that water stress decreased germination and germ tube elongation of *L. taurica* on pepper plants.

Chapter (2)

Materials and Methods

2.1 Plant Material

The seeds of tomato (*Lycopersicon esculentum* Mill.) cultivars used in cell and tissue culture were: Moneymaker (Brooker Seeds, Steaford, U.K.) and Rootstock (J.W. Moles and Sons, Essex, U.K.). Seeds of *Lycopersicon chilense* were obtained as a gift from Prof. C.M. Rick of the Tomato Genetic Stock Center at the University of California, Davis (U.S.A.). Five accessions were received which had shown some resistance to powdery mildew (Rick, personal communication). Two of these, LA2747 and LA458 were used in the present study. Breeder seeds of tomato were also received from Prof. A.A. Geneif (Horticultural Research, Wad Medani, Sudan) to be screened against powdery mildew. Tomato cultivar Hawaii 7998 (resistant to *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) was a gift from Dr. L.W. O'Garro, School of Biology, University of the West Indies at Barbados.

Pepper (*Capsicum annuum* L.) cultivars Earlycalwonder (ECW) and Earlycalwonder-10R (ECW-10R) were received from Dr. J. Clarkson, Plant Biology, University of Bath, United Kingdom.

All seeds were stored at 4 °C. In addition, clones of the wild accessions of tomato were also maintained as stock plants in a greenhouse (minimum day/night temperature of 24/18 °C, natural day light).

2.2 Propagation of Plant Material in the Greenhouse

Tomato seeds were sown in half trays filled with Fison F2 compost and germinated in a greenhouse at a minimum temperature of 25 °C in the dark. Germination took place between 5-7 days, after which seedlings were allowed to

grow for one week before transplanting into 2 litre plastic pots containing Fison M2 compost and grown in a greenhouse (in natural daylight) at a minimum temperature of 24 °C. Liquid feed of NPK (20:20:20) was added to the irrigation water at the rate of (100 g l^{-1}) using a Cameron Diluter. This propagation method was also used for peppers, but the germination time of the pepper seeds was 12-14 days.

2.3 Tissue Culture

2.3.1 Culture Vessels

Callus induction and plant regeneration were carried out in 5 and 9 cm diameter plastic petri dishes (Sterilin Ltd.), while 60 ml plastic vials (Sterilin Ltd.) were used for tomato regenerant growth and maintenance. Rooting was induced on sterilized filter paper in 50 ml glass test tubes (Pyrex) containing 10 ml liquid MS medium. 250 ml Erlenmeyer flasks containing 100 ml liquid B5 medium were used for tomato suspension culture maintenance. Glass test tubes and plastic vials were covered with plastic or aluminium caps, while petri dishes were sealed with Parafilm M. (American Can Co.).

2.3.2 Culture Media

The culture media used were Murashige and Skoog (1962) (MS) basal medium supplied by Flow Laboratories (Scotland) or Gamborg et al. (1968) (B5) medium, Sigma, USA. Unless otherwise stated, the carbon source was sucrose (3%, w/v).

Heat-stable plant growth substances such as benzylaminopurine (BAP) were added before autoclaving, but the heat-labile ones such as indole acetic acid (IAA) were filter sterilized through a membrane filter of pore size 0.2 μm (Sartorius Ltd.) and added to the medium as it cooled (50 °C).

The pH of the media was adjusted to 5.8 with 1.0/0.1 M NaOH or 1.0/0.1 M HCl and the media were gelled with 0.8% (w/v) agar (Oxide No. 3). All media were autoclaved for 15 minutes at 1.87 bar and 120 °C.

2.3.3 Surface Sterilization of Tomato Seeds

Tomato seeds were agitated in 1.0% sodium hypochlorite (14% w/v available chlorine) for 5 minutes. A few drops of polyoxyethylene sorbitol mono-oleate were added (Tween 80 Sigma, U.S.A.). The seeds were then washed in several changes of sterile double distilled water, and germinated on 20 ml of MS medium in 9 cm petri dishes. Cultures were incubated in a culture room with 16 hours day length, at 25 °C and 50 $\mu E\ m^{-2}\ s^{-1}$ light. Germination took place after one week.

2.3.4 Culture Incubation Conditions

Semi-solid cultures were incubated in a culture room at 25 ±1 °C, 16 hours photoperiod and light intensity 50.0 $\mu E\ m^{-2}\ s^{-1}$.

2.3.5 Tomato Suspension Culture

Callus of tomato cv. Rootstock *(L. hirsutum* X *L. esculentum)* was obtained from BP Research Laboratories, Sunbury, U.K. The callus was initially established from hypocotyl explants and maintained on a medium containing B5 salts and vitamins supplemented with 1.0 mg l^{-1} 2,4-D, 2% sucrose and adjusted to pH 5.8 and solidified by the addition of 0.8% agar. The callus was subcultured at monthly intervals.

The medium used for suspension culture was B5 salts and vitamins with 2% sucrose, 0.1 mg l^{-1} 2,4-D, and 0.5 mg l^{-1} of both BAP and NAA. The suspension culture was initiated by dividing 5 g callus by a glass rod and

transferring it into 100 ml B5 medium in screw top 250-ml Erlenmeyer flasks. These flasks were incubated at 25 ±1 °C, 16 hours day and light 150.0 μE m^{-2} s^{-1} in an orbital shaker incubator at 120 rpm (Gallenkamp).

The suspension culture was subcultured every two weeks when packed cell volume (pcv) was determined by spinning 10 ml of the original suspension culture at 3000 g in a calibrated centrifuge tube. Five percent (pcv) suspension was sub-cultured into 250-ml Erlenmeyer flasks containing 100 ml of B5 medium. Viability of the tomato cells was assessed by fluorescein diacetate (FDA) Widholm (1972). The stock solution was prepared by dissolving 5.0 mg of FDA in 1.0 ml acetone. This was further diluted in B5 medium (1:20 v/v) before one drop was added to the cell suspension on a glass slide and observed under a fluorescence microscope with blue light (530 nm).

2.4 Anatomy of Tomato Hypocotyl Explants

The procedures used for fixation, dehydration, infiltration, embedding, sectioning and staining were modified from Taylor (1991).

a. Fixation:

Formalin/Acetic Acid (FAA) (Glacial Acetic Acid: Formalin; 70% and ethanol 5:5:90 mls) was used for specimen fixation for a minimum of 48 hours.

b. Dehydration:

The specimens were then passed through an increasing alcohol and 2-methylpropane-2-ol (TBA) sequence, starting at step 6 (Table 2.1) for 12 hours in each step. Table 2.2 shows the TBA dilution series.

c. Infiltration:

Molten paraffin wax was poured into the TBA vials and kept in a wax oven at 70 °C. Four changes of wax, 4-5 hours each, ensured TBA evaporation and complete infiltration.

Table (2.1): The sequence of alcohol and TBA concentrations used for the dehydration of fixed specimens.

Stage	Alcohol	Time (hours)
1	2.5% C_2H_5OH	12
2	5% C_2H_5OH	12
3	10% C_2H_5OH	12
4	20% C_2H_5OH	12
5	30% C_2H_5OH	12
6	50% C_2H_5OH	12
7	TBA1*	12
8	TBA2*	12
9	TBA3*	12
10	TBA4*	12
11	TBA5*	12
12	Sat. soln. of erythrosin in 100% TBA	12
13	100% TBA	12

TBA = 2-methylpropane-2-ol

* See Table (2.2) for TBA dilution series.

Table (2.2): TBA Dilution Series (mls.)

Series	H_2O	C_2H_5OH (95%)	TBA	C_2H_2OH (100%)
TBA1	50	40	10	--
TBA2	30	50	20	--
TBA3	15	50	35	--
TBA4	--	50	50	--
TBA5	--	--	75	25

d. Embedding:

Rectangular boats were constructed from card and filled with molten wax. Four specimens were transferred to these with forceps and oriented perpendicular to the boat's longitudinal axis. The surface of the wax was then allowed to cool before immersion in cold water.

e. Sectioning:

A Reichert microtome was used to cut 6 μm sections from the specimens. The ribbons were expanded by floating on hot water and transferred to slides smeared with Haupt's solution adhesive (1.0 g gelatin, 2.0 g phenol and 15 ml glycerol dissolved in 100 ml distilled water at 60 °C). The slides were then allowed to dry.

f. Staining:

The slides were taken through a rehydration, staining and dehydration procedure (Table 2.3), mounted with DPX-mountant, dried overnight and examined with a light microscope.

2.5 *Xanthomonas campestris* pv. *vesicatoria*

2.5.1 Bacterial Strains and Media

Five strains of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) were kindly received from Dr. D. Dymock and Dr. J. Clarkson, School of Biology, University of Bath, U.K. Two mutants E1141 and E1004 were derived by EMS mutagenesis of the wild-type pathogenic strain E3 mutated in *hrp* gene, and they caused no symptoms on susceptible cultivars of tomato and pepper and failed to produce a hypersensitive reaction (HR) on resistant pepper cv. ECW-10R (Clarkson, personal contact). Pathogenicity, and the ability to induce an HR were restored in E1141 and E1004 by complementation with the plasmid p6AD4, which is a cosmid pLAFR3 containing ~ 22 kb inserted from *Xcv* 2595 which complements the

Table (2.3): Procedure for Staining Sections with Toluidine Blue

Solution	Time (min.)
Three passes in 100% Histoclear	3 each
50/50 Histoclear/100% C_2H_5OH	3
100% C_2H_5OH	3
95% C_2H_5OH	3
70% C_2H_5OH	3
50% C_2H_5OH	3
30% C_2H_5OH	3
Distilled Water (H_2O)	3
1% Toluidine Blue in H_2O	0.75
Two passes in H_2O	1 each
30% C_2H_5OH	1
50% C_2H_5OH	0.5
70% C_2H_5OH	0.5
95% C_2H_5OH	0.5
100% C_2H_5OH	0.5
50/50 Histoclear/ 100% C_2H_5OH	1
Five passes in 100% Histoclear	5, or more each

mutant M461 Seal et al. (1990). this plasmid is resistant to tetracycline (D. Dymock, personal communication).

The wild-type strain and *hrp* mutants were routinely cultured on nutrient yeast glycerol agar (NYGA, Appendix 2) plates, while the complemented pathogenic mutants were grown on NYGA supplemented with filter sterilized tetracycline (12.5 mg l^{-1}).

2.5.2 Long Term Storage of Cultures

Bacterial strains were plated on NYGA plates. One colony from each strain was cultured overnight in 10 ml NYGB on an orbital shaker at 30 °C. 20% (v/v) sterilized glycerol was then added to the cultures, mixed thoroughly and frozen in sterilized Eppendorfs at -70 °C. Whenever required, the cultures were thawed at room temperature and streaked onto fresh NYGA.

2.5.3 Preparation of Xcv Inoculum

Xanthomonas campestris pv. *vesicatoria* strains were streaked on NYGA plates and grown for 2-3 days at 30 °C. NYGB cultures (10 ml in 30 ml plastic universal) were initiated from single colonies of the NYGA plated strains and incubated on a gyratory shaker (120 rpm) at 30 °C overnight. Bacterial cells were harvested by centrifugation of the broth at 3000 g for 15 minutes and the supernatant was discarded. Inoculum was prepared by washing the cells twice and resuspending the pellet in sterile distilled water. From a standard curve relating OD at 600 nm to the number of colony forming units (cfu), the suspension was adjusted to a density equivalent to 10^8 cfu ml^{-1}. Inoculum that contained approximately 10^6 viable cells per ml was prepared by appropriate dilution in distilled water. Heat-killed cells prepared by heating cultures to 70 °C for one hour in a water bath served as the control. This was done after the bacterial numbers were adjusted.

2.5.4 Inoculation of Plants with *Xcv*

Test plants (tomato or pepper) were grown in the greenhouse (Section 2.2) until the first 4 leaves were fully expanded and then moved to a temperature controlled Fisons growth chamber (25 °C) in which all experiments were conducted. Light (180-250 $\mu E\ m^{-2}\ s^{-1}$) was supplied for all plants from "Cool-White" fluorescent tubes placed approximately 30 cm above the plants. Intermittent light was used herein to give 16 hours exposure to light followed by 8 hours darkness. The relative humidity was 88-92%.

All inoculations were accomplished by leaf infiltration (Klement, 1963) with bacterial suspensions using a hypodermic syringe without a needle. Liquid was forced into the leaf, through small cuts (2mm diameter) on the lower epidermis made by a needle, resulting in the part of the leaf in which the air in the intercellular space was replaced by liquid becoming visibly water-soaked. In most of the inoculations the total leaf was water soaked, but in those instances when the leaf was young, only a portion of the leaf (about 1 cm wide and 2 cm long) on both sides of the midrip was water soaked. The leaves were rinsed with distilled water to remove bacterial cells on the leaf surface.

2.5.5 Electrolyte Leakage from *Xcv* Infected Leaves

In each test, 4 leaves of different position and age were selected on each of the plants to be utilized. Tissue samples were taken from these leaves at different positions on the leaf surface. This method of sampling was designed to compensate for differences between plants and leaf age on any one plant. The tissue samples were taken by cutting out discs (4 mm diameter) with a cork borer. Fifteen discs per replicate were weighed and placed in 30 ml plastic universal containing 7.5 ml of distilled water. The discs were kept submerged with a plastic screen and conductivity was measured. Immediately after the first reading, the leaf discs in water were placed in a desiccator connected with a

pump which reduced the pressure after 3-5 minutes and the leaf discs were infiltrated when the pressure was released. The discs were shaken on an orbital shaker at 120 rpm, temperature 25 °C and transferred to 25 °C water bath, until the second reading was taken after 1 hour. The difference in readings represented electrolyte leakage/hour. These readings were expressed as a percentage of cell leakage of the total ions present in non-inoculated leaf discs which were first frozen at -70 °C for 24 hours to release all cell contents.

2.5.6 Determination of Growth of *Xcv* in Leaves of Pepper or Tomato Plants

One ml of the bacterial suspension prepared in Section 2.5.5 for cell leakage, was taken for the assessment of bacterial growth *in vivo* by a dilution-plate procedure. Ten fold serial dilutions were made in sterile water and 100 µl aliquots of the approriate dilutions were placed onto NYGA plates replicated three times. The plates were incubated at 30 °C for 48-72 hours and typical yellow bacterial colonies were counted. Viable bacterial populations were expressed as colony forming unit per gm fresh weight (cfu/gm fr.wt.). The first leaf samples were always taken within 2 hours of inoculation. Sampling usually continued every day for 4 days and then at 2 day intervals for 8-10 days, unless the leaves withered so as to prevent further determinations. The plants were observed for symptoms development before the leaf sampling.

2.6 Powdery Mildew of Tomato

2.6.1 Source and Maintenance of Powdery Mildew

Powdery mildew of tomato (*Erysiphe cichoracearum*) was obtained from Dr. J. Fletcher (ADAS, Wye, Kent, U.K.). The disease was maintained on 3-5 week-old tomato seedlings (propagated as described in Section 2.2). The seedlings were placed in a cylinderical spore settling tower made of cardboard and inoculated by putting the infected tomato leaves over the upper end of the spore

settling tower and tapping off the conidia. By this inoculation method single conidia were evenly distributed over the leaf surface. The inoculated tomato seedlings were placed on a bench in the greenhouse (minimum day/night temperature of 24/18 °C, natural day light), and enclosed in an open-top clear polythene frame (1 m^3). Several of these partially protected frames were employed. Disease symptoms showed on the upper leaf surface after about one week. New tomato seedlings were taken into the wooden frame every 2-3 weeks and the same inoculation method was applied using the infected tomato plants as an inoculum source.

For experimental work following inoculation, tomato plants were incubated in a Fisons growth chamber 24 °C, 16 hours photoperiod (180-250 μE m^{-2} s^{-1} light) and relative humidity of 88-92%. These plants were shaken 24 hours before the beginning of each experiment to dislodge old conidia and thereby provide inoculum of relatively uniform age. The inoculum density was monitored by placing a Vaseline-coated microscopic slide among the plants or the leaves.

2.6.2 Effect of Temperature and Humidity on Germination of Powdery Mildew Conidia and Germ Tube Length

The method for determining the effect of relative humidity (rh) on conidia germination was adopted from Manners and Hossain (1963). Staining blocks were used as humidity chambers because repeated microscopic observations could be made through the glass lid without opening the chamber. To ensure air-tight seals, the upper surface was smeared with Vaseline. Two rectangular plastic fragments (2 mm thick) were stuck to the lower surface of the lid to provide a space between the conidia and the lid (Figure 2.1). The desired rh (5-100%) were obtained from solutions of potassium hydroxide (Salmon, 1951; Unwin, 1980). Potassium hydroxide concentrations of 7, 12, 19, 30, 38 and 47 g were dissolved

in 100 distilled water to give 95, 90, 80, 60, 40 and 20% rh respectively. Distilled water and solid sodium hydroxide (0.5 g per chamber) were used to give 100% and 5% rh respectively.

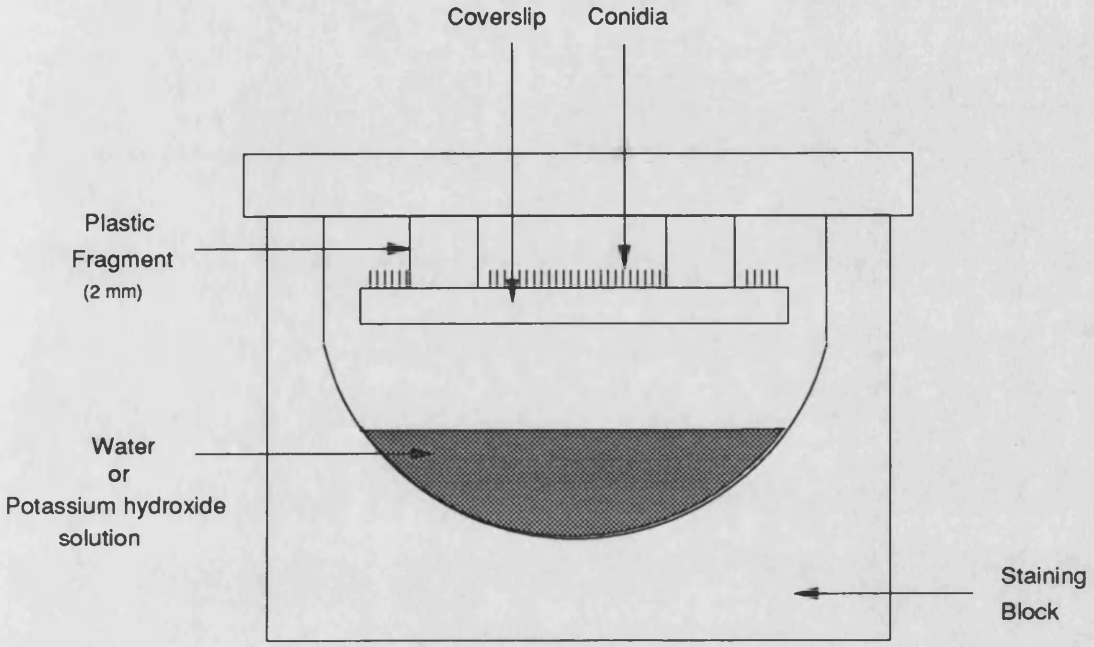


Figure 2.1: Humidity chamber employed for germination test of powdery mildew conidia at 5-100% relative humidity

Conidia for the germination test were collected from diseased plants which were grown in a growth chamber (described in Section 2.6.1). Coverslips were arranged on a black cloth and diseased plants were shaken over a settling tower (Section 2.5.1). Cover slips with good coverage of conidia were selected and cemented with Vaseline to the rectangular plastic fragments on the lid of the chamber. The chambers were prepared in advance so that not more than 20 minutes elapsed between the collection of the spores and the commencement of incubation. Three replicates for each humidity were incubated in a dark incubator at 10, 15, 20, 25, 30, 35 and 40 °C each time.

The germinated conidia were assessed after 24 hours. A conidium was considered germinated when its germ tube length was longer than its breadth. For the determination of percentage germination, 300 conidia from each chamber were counted from three fields under the microscope selected randomly, each containing approximately 100 conidia. In tests at 100% rh., fields relatively free from condensation were selected. Germ tube length was measured with the aid of an ocular micrometer, under high power. From each coverslip twenty five germ tubes were chosen randomly and measured. The experimental work on the germiation of conidia was carried out using the same diseased plants as a source of inoculum to minimize the differences between inoculum batches.

2.6.3 Fixation and Preparation of Powdery Mildew for Light Microscopy

Infected leaf discs were excised and laid adaxial surface up on a filter paper bed moistened with fixative (3 absolute ethanol: 1 glacial acetic acid; v:v). The fixative migrated through the abaxial surface, but it did not directly contact the upper surface, ensuring that conidia were not displaced or washed off by the process. After 48 hours, bleached segments were transferred adaxial surface up to a filter paper pad moistened with lactophenol which softened and cleared the tissues (24 hours) (Carver and Adaigbe, 1990), and stained with calcofluor for 5 min. before microscopic examination (Cohen et al., 1987).

2.6.4 Staining and Microscopy for the Assessment of Host-pathogen Interaction

2.6.4.1 Fluorescence Microscopy

For microscopy, leaf segments were laid adaxial surface up on a glass slide smeared with lactophenol to give liquid continuity between the slide and abaxial leaf surface. A droplet of aqueous 0.01%, calcofluor (calcofluor white M2R, disodium salt of 4, 4-bis (4-anilino-6-diethyl-amino-s-triazin-2-yl amino) -2,2-

stilbene disulfonic acid) (Polysciences Inc., Warrington, PA) solution was applied to each inoculated site. No coverslip was applied as this could have displaced loosely attached fungal structures. Consequently the specimens were examined using a 'no coverslip' objective. Episcopic fluorescence microscopy with an excitation wavelength of 435 nm, dichromatic mirror with 455 nm cut off, and 475 nm barrier filter was used to examine the fungal structures.

2.6.4.2 Scanning Electron Microscopy

The specimens were prefixed in 2% glutaraldehyde in 0.1 M Sorensens phosphate buffer pH 6.9 at room temperature (RT) for 4 hours. Then the specimens were washed in 0.2 M Sorensens (pH 6.9) for 15 min. at RT, followed by further fixation in 1% osmium (OsO_4) in 0.18 M Sorensens phosphate buffer (pH 6.9) for one hour at RT and washed in deionized distilled water overnight at 4 °C. They were dehydrated in a graded ethanol series at RT and finally placed into 100% dry alcohol and acetone for 15 min. each at RT. They were then dried in a critical-point dryer with liquid CO_2, sputter-coated with gold to a thickness of about 20 nm and examined with T-330 JEOL scanning electron microscope.

2.7 Microscopes and Photography

An Olympus BH-2PC microscope was used for bright field, phase contrast and ultraviolet examination. Photomicrographs were taken with an attached Olympus OM-2 camera. Photographs of cultures or infected plant tissues were taken with an Olympus OM-2 camera. Kodachrome 64 or Kodak panatomix 32 film were used to obtain colour transparancies and black and white prints respectively.

2.8 Statistical Analysis

The data were analysed using the split-plot or completely randomised designs and the means were separated by Duncan Multiple Range Test at 5%, Student's t-test or partial testing.

Chapter (3)

Tissue Culture of Tomato

3.1 Results of Tissue Culture of Tomato

Seeds of tomato cvs Moneymaker and Rootstock were sterilized with 1.0% sodium hypochlorite (Section 2.3.3), germinated on MS medium prepared and sterilized as described in Section 2.3.2, and all cultures were incubated in a culture room described in Section 2.3.4.

3.1.1 Morphogenesis of Tomato cv. Moneymaker Explants Cultured on MS Medium Supplemented with BAP and NAA

One month old tomato seedlings were divided into cotyledons, stem internodes and hypocotyl explants. Cotyledons were separated and divided into two. Leaf segments including a portion of the midvein were excised and plated abaxial side up onto MS medium supplemented with benzylaminopurine (BAP) and naphthalene acetic acid (NAA). Four levels of BAP (0, 2.2, 4.4 and 8.8 μM) were used in a factorial combination with NAA at 0, 2.7, 5.4, and 10.8 μM. Forty explants per treatment were incubated in the culture room described in section 2.3.4. Shoot and root regeneration were assessed visually 4 weeks after the initiation of cultures. The Amount of callus was also scored using the following scale: * = small; ** = moderate and *** large. Callus or shoots produced were excised and maintained on the same medium as that used for their induction.

3.1.1.1 Callus Induction

Generally, compact callus was produced in all combinations of the plant growth substances on a high frequency of all explants. NAA alone induced a small amount of callus at the cut surfaces irrespective of the concentration used (Figures 3.1-3.4). When a low concentration of NAA was combined with BAP, the amount of callus increased with increasing levels of BAP (Figures 3.1-3.4). With the exception of hypocotyl explants, the combination of high levels of NAA/BAP produced a large amount of callus. Cytokinin produced small to moderate amount of green callus on all explants except hypocotyl explants treated with the highest level of BAP which produced a large mass of callus (Figures 3.1-3.4).

3.1.1.2 Root Regeneration

In response to NAA alone, a large percentage of explants produced roots (Figures 3.1-3.4). Three weeks after the initiation of cultures, roots were produced on a considerable area of the leaf and cotyledon explants cultured on high concentrations of NAA, but on hypocotyl and stem internode explants the production of the roots was mainly at the cut surface. A small number of the hypocotyl and stem internode explants produced roots when treated with low levels of NAA combined with BAP but combinations with other levels of NAA failed to produce roots (Figures 3.1 and 3.3). In contrast, the leaf explants produced roots on all NAA/BAP media although the frequency of explants producing roots was lower when higher concentrations of BAP were used (Figure 3.2). A similar pattern was found with cotyledon explants, but in the presence of BAP, less than 20% of the explants responded (Figure 3.2). No roots were observed on explants treated with BAP alone (Figures 3.1-3.4).

Figure 3.1 and 3.2 : Morphogenesis of hypocotyl and cotyledon explants of tomato cv. Moneymaker cultured on MS medium supplemented with BAP and NAA

Forty explants per treatment were cultured on MS medium supplemented with BAP and NAA, and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1} for 4 weeks.

Key:

Shoot regeneration

Root regeneration

Callus induction

Amount of callus

* Small callus

** Moderate callus

*** Large callus

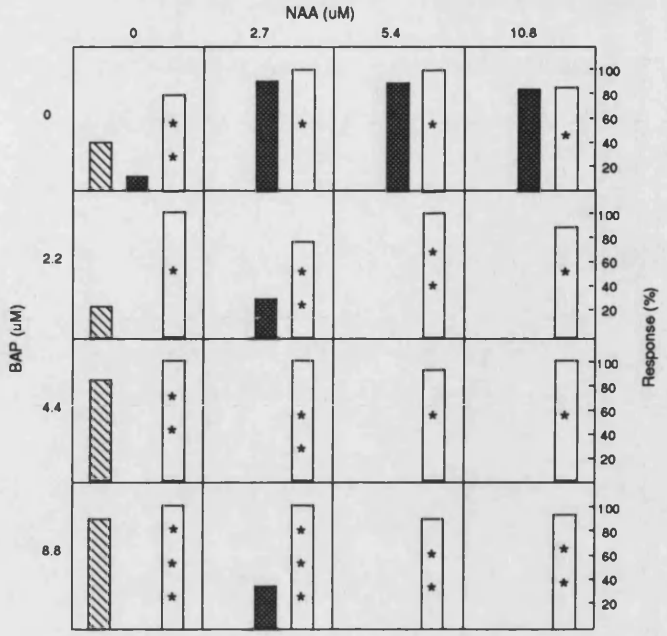


Figure 3.1 :Morphogenesis of tomato cv. Moneymaker hypocotyl explants on MS medium supplemented with BAP and NAA

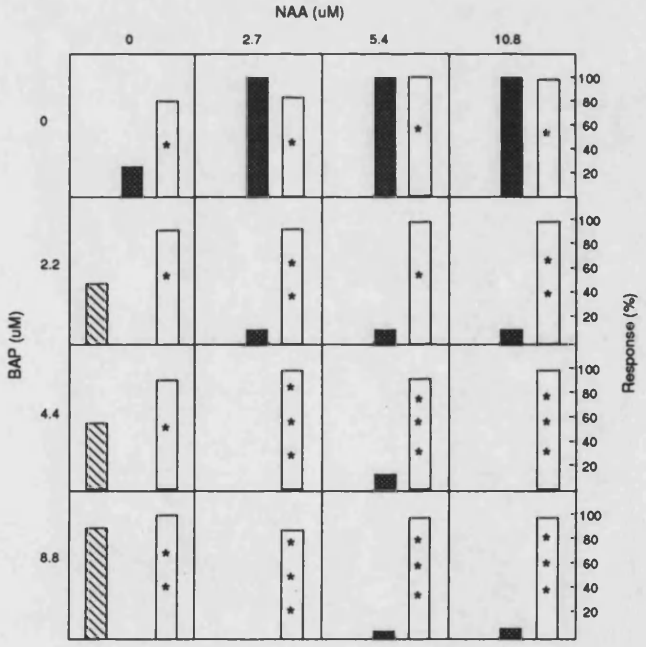
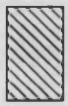


Figure 3.2 : Morphogenesis of tomato cv. Moneymaker cotyledon explants on MS medium supplemented with BAP and NAA

Figure 3.3 and 3.4 : Morphogenesis of stem internode and leaf explants of tomato cv. Moneymaker cultured on MS medium supplemented with BAP and NAA

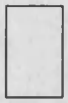
Forty explants per treatment were cultured on MS medium supplemented with BAP and NAA, and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 $\mu E\ m^{-2}\ s^{-1}$ for 4 weeks.

Key:



Shoot regeneration

Root regeneration



Callus induction

Amount of callus

* Small callus

** Moderate callus

*** Large callus

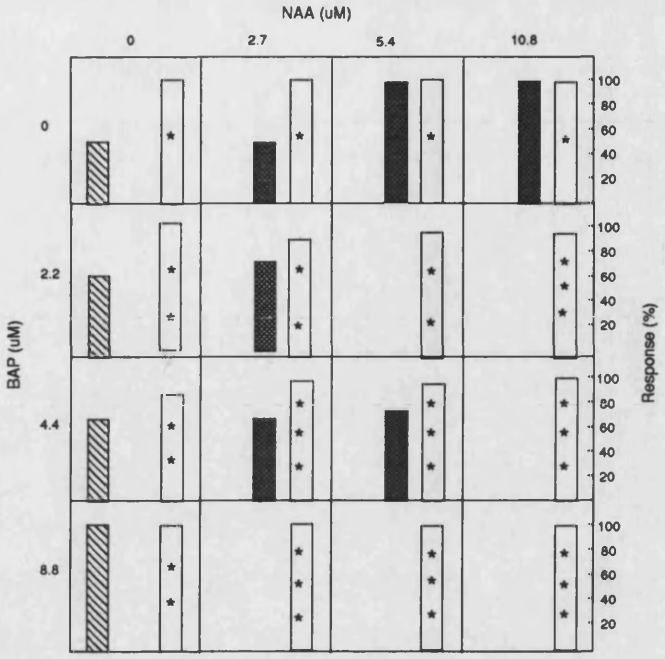


Figure 3.3 : Morphogenesis of tomato cv. Moneymaker stem internode explants on MS supplemented with BAP and NAA

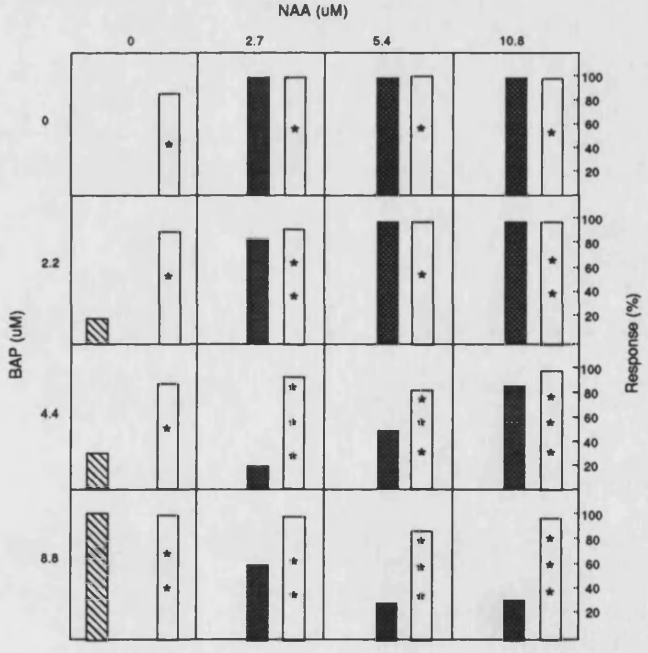


Figure 3.4 : Morphogenesis of tomato cv. Moneymaker leaf explants on MS medium supplemented with BAP and NAA

3.1.1.3 Shoot Regeneration

Shoot regeneration was observed in all treatments with BAP and no NAA, and the frequency of responding explants increased with the increase in the cytokinin concentration (Figures 3.1-3.4). Hypocotyl and stem internode explants produced shoots on MS medium with no BAP in the medium (Figures 3.1 and 3.3). The morphogenetic response of hypocotyl explants on BAP and NAA combinations is shown in Plate 3.1. No shoots were produced on explants cultured on either NAA or any combination of BAP and NAA (Figures 3.1-3.4). The number of shoots produced per explant was similar on all explants cultured on BAP $\leq$ 4.4 μM, but it was significantly higher ($p < 0.05$) on the highest BAP concentration (10.8 μM) (Table 3.1). However, the explant type had no effect on the number of shoots.

3.1.2 Morphogenesis of Tomato cv. Moneymaker Explants Cultured on MS Medium Supplemented with BAP and 2,4-D

In this experiment the same BAP levels were used as in the previous experiment (Section 3.1.1), but NAA was replaced by 2,4-D. Different explants of tomato cv. Moneymaker (hypocotyl, cotyledon, stem internode, and leaf) were cultured on four levels of BAP (0, 2.2, 4.4 and 8.8 μM) which were combined with 2,4-D at 0, 2.25, 4.5, and 9.0 μM. Forty explants per treatment were incubated in the culture room described in Section 2.3.4. Regeneration of shoots and roots were assessed visually 4 weeks after the initiation of the cultures. The amount of induced callus was assessed using the following scale: * = small; ** = moderate and *** large. Callus or shoots produced were excised and maintained on the same medium as that used for their induction.

Plate 3.1: Morphogenesis of tomato cv. Moneymaker hypocotyl explants on MS supplemented with BAP and NAA 4 weeks after culture initiation.

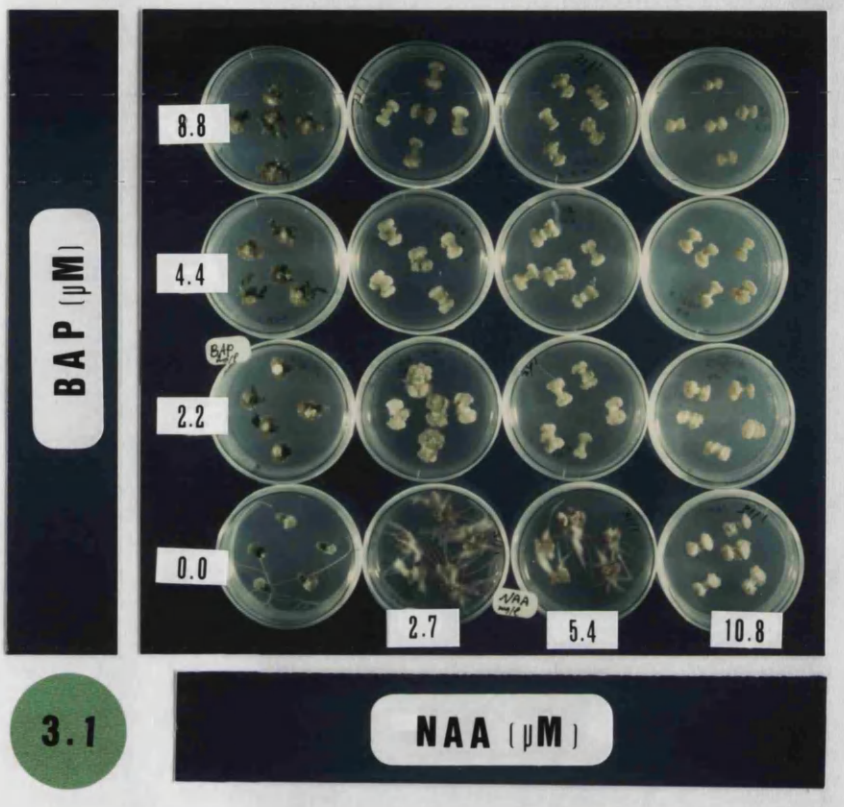

BAP (μM)
8.8
4.4
2.2
0.0
2.7
5.4
10.8
3.1
NAA (μM)

Table 3.1 Number of shoots per responding explant of tomato cv. Money-maker on MS supplemented with BAP and NAA

Plant growth substance μM		Mean number of shoots per responding explant			
BAP	NAA	Hypocotyl	Cotyledon	Stem internodes	Leaves
0	0	1.0 b	0	1.0 b	0
0	2.7	0	0	0	0
0	5.4	0	0	0	0
0	10.8	0	0	0	0
2.2	0	1.0 b	1.0 b	1.0 b	1.0 b
2.2	2.7	0	0	0	0
2.2	5.4	0	0	0	0
2.2	10.8	0	0	0	0
4.4	0	1.0 b	1.0 b	1.07 ± 0.05 b	1.0 b
4.4	2.7	0	0	0	0
4.4	5.4	0	0	0	0
4.4	10.8	0	0	0	0
8.8	0	1.61 ± 0.08 a	1.19 ± 0.06 a	1.38 ± 0.08 a	1.29 ± 0.07 a
8.8	2.7	0	0	0	0
8.8	5.4	0	0	0	0
8.8	10.8	0	0	0	0

Forty hypocotyl, cotyledon, stem internode and leaf explants of tomato cv. Moneymaker per treatment were cultured on MS medium supplemented with BAP and NAA, incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1} for 4 weeks.

Values within a column followed by the same letter are not significantly different at 5% according to student's t-test.

3.1.2.1 Callus Induction

Callus was produced on a high percentage of explants of each treatment, but the amount of callus varied in response to the different combinations of BAP and 2,4-D (Figures 3.5-3.8). A large amount of callus was produced on the hypocotyl explants cultured on all concentrations of 2,4-D (Figure 3.5 and Plate 3.2). Friable callus was produced on hypocotyl and stem internode explants cultured on 4.5 μM 2,4-D. However, leaf explants produced small and compact callus on all 2,4-D concentrations (Figure 3.8). The amount of callus produced on cotyledon explants increased with the increasing 2,4-D concentration (Figure 3.6). The combination of a low concentration of 2,4-D and BAP produced large and compact callus on stem internode and leaf explants, as did the combination of high levels of both plant growth substances (Figures 3.7 and 3.8).

3.1.2.2 Root Regeneration

Roots were regenerated on 55% of the cotyledon explants when cultured on MS without plant growth substances, but more were produced on other explants i.e. hypocotyl, leaf and stem internode (Figures 3.5-3.8). No root initiation was found on any explant cultured on MS medium supplemented with BAP and/or 2,4-D (Figures 3.5-3.8).

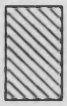
3.1.2.3 Shoot Regeneration

The replacement of NAA with 2,4-D did not improve the frequency of shoot regeneration on tomato explants. Shoot regeneration was only noted on explants cultured on cytokinin media. The number of explants producing shoots increased with the increasing BAP concentration (Figures 3.5-3.8 and Plate 3.2). High BAP concentrations were required to produce shoots on leaf explants, in contrast to stem

Figure 3.5 and 3.6 : Morphogenesis of hypocotyl and cotyledon explants of tomato cv. Moneymaker cultured on MS medium supplemented with BAP and 2,4-D

Forty explants per treatment were cultured on MS medium supplemented with BAP and 2,4-D, and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1} for 4 weeks.

Key:



Shoot regeneration

Root regeneration

Callus induction

Amount of callus

* Small callus

** Moderate callus

*** Large callus

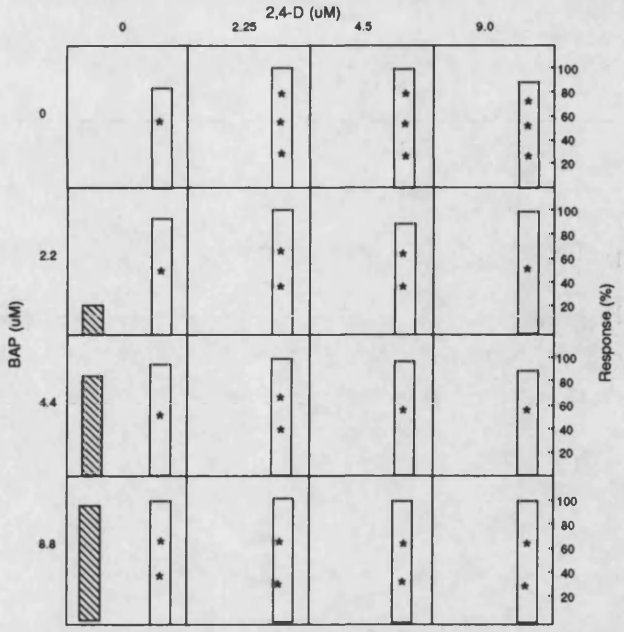


Figure 3.5 : Morphogenesis of tomato cv. Moneymaker hypocotyl explants on MS medium supplemented with BAP and 2,4-D

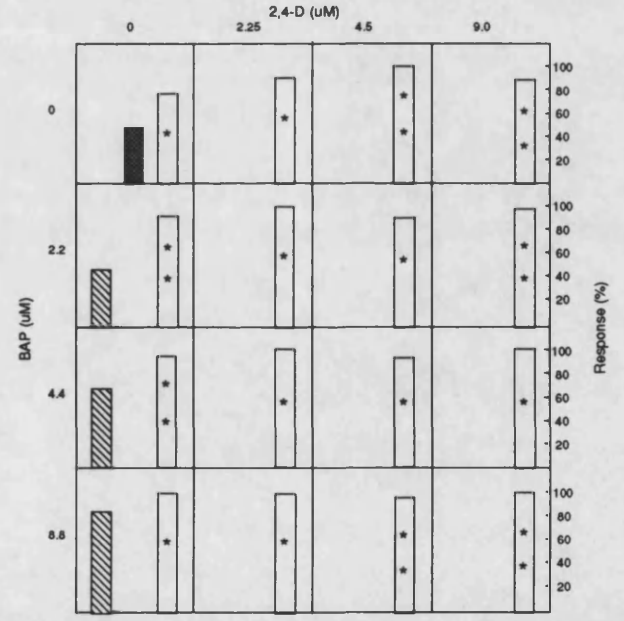


Figure 3.6 : Morphogenesis of tomato cv. Moneymaker cotyledon explants on MS medium supplemented with BAP and 2,4-D

Figure 3.7 and 3.8 : Morphogenesis of stem internode and leaf explants of tomato cv. Moneymaker cultured on MS medium supplemented with BAP and 2,4-D

Forty explants per treatment were cultured on MS medium supplemented with BAP and 2,4-D, and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1} for 4 weeks.

Key:

Shoot regeneration

Root regeneration

Callus induction

Amount of callus

* Small callus

** Moderate callus

*** Large callus

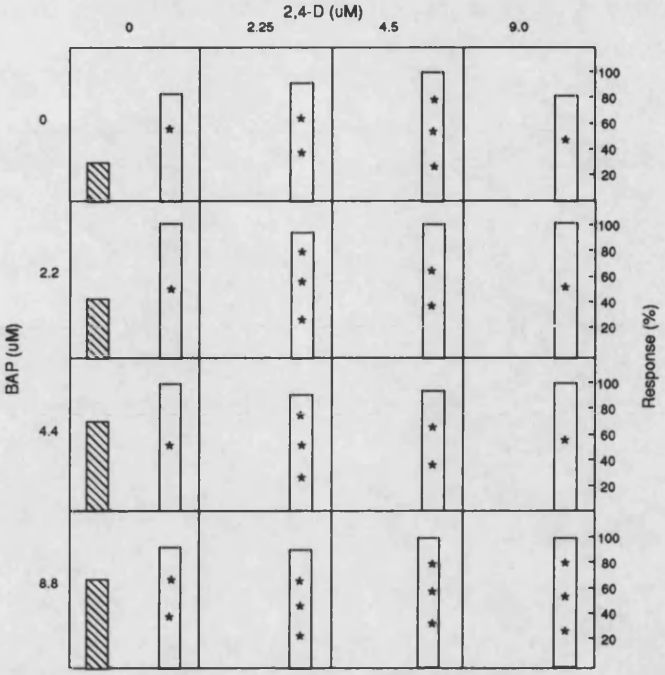


Figure 3.7 : Morphogenesis of tomato cv. Moneymaker stem internode explants on MS medium supplemented with BAP and 2,4-D

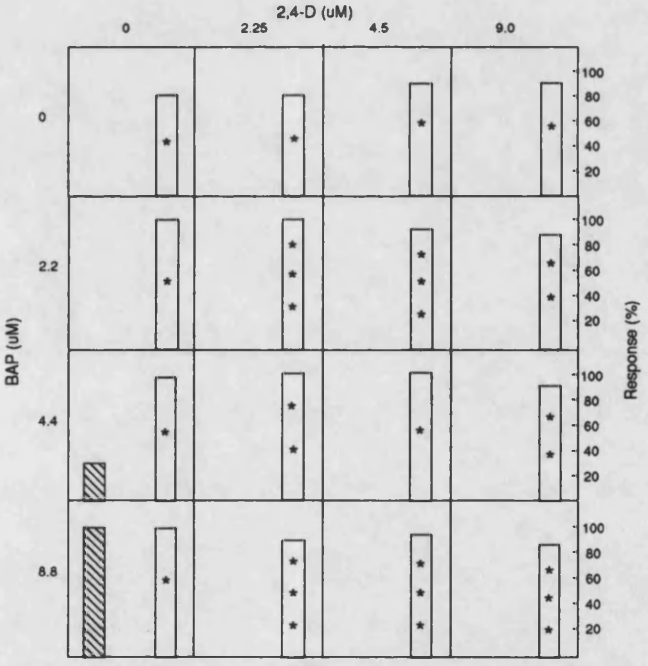
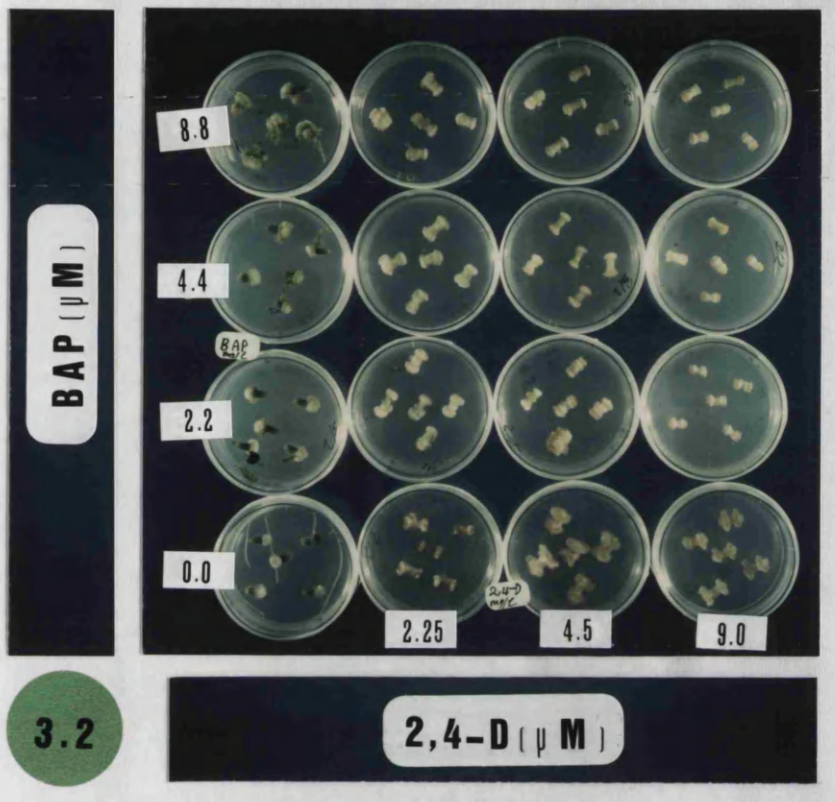


Figure 3.8 : Morphogenesis of tomato cv. Moneymaker leaf explants on MS medium supplemented with BAP and 2,4-D

Plate 3.2: Morphogenesis of tomato cv. Moneymaker hypocotyl explants on MS supplemented with BAP and 2,4-D 4 weeks after culture initiation.


BAP (μM)
8.8
4.4
2.2
0.0
2.25
4.5
9.0
3.2
2,4-D (μM)

internode explants which were able to regenerate shoots on media free of plant growth substances (Figures 3.7 and 3.8). There was no significant difference in the number shoots produced per responding explant between the different explants on any given medium (Table 3.2). The number of shoots produced on explants cultured on the highest BAP concentration was significantly higher in comparison to those on the lower concentrations (Table 3.2).

3.1.3 Morphogenesis of Tomato cv. Moneymaker Shoot Tips cultured on MS Medium Supplemented with BAP and NAA

The morphogenetic response of shoot tip explants of tomato cv. Moneymaker was investigated on MS medium supplemented with BAP (0, 2.2, 4.4, 6.6 and 11.0 μM) and NAA (0, 0.5, 2.2, 4.3, and 5.4 μM). Twenty shoot tip explants per treatment, taken from 2 weeks-old seedlings, were cultured in the culture room described in Section 2.3.4, for 4 weeks before their morphogenetic response was assessed.

Shoot regeneration was observed on a high percentage of explants cultured on MS medium free from plant growth substances and when this medium was supplemented with BAP (Figure 3.9). Shoots were also regenerated on a single combination of BAP/NAA (11.0/0.5 μM), but other combinations failed to induce the regeneration of shoots on the leaf explants (Figure 3.9). Only single shoots were regenerated per responding explant. These were apical meristem derived shoots. Roots were regenerated on explants cultured on NAA alone, and NAA in combination with low BAP concentrations (Figure 3.9). The number of explants producing roots decreased when the concentration of both plant growth substances was increased, and no roots were produced when high levels of BAP and NAA were combined

Table 3.2 Number of shoots per responding explant of tomato cv. Moneymaker on MS supplemented with BAP and 2,4-D

Plant growth substance µM		Mean number of shoots per responding explant			
BAP	2,4-D	Hypocotyl	Cotyledon	Stem internodes	Leaves
0	0	0	0	1.0 b	0
0	2.25	0	0	0	0
0	4.5	0	0	0	0
0	9.0	0	0	0	0
2.2	0	1.0 b	1.0 b	1.0 b	0
2.2	2.25	0	0	0	0
2.2	4.5	0	0	0	0
2.2	9.0	0	0	0	0
4.4	0	1.04 ± 0.03 b	1.0 b	1.15 ± 0.07 ab	1.0 a
4.4	2.25	0	0	0	0
4.4	4.5	0	0	0	0
4.4	9.0	0	0	0	0
8.8	0	1.37 ± 0.10 a	1.16 ± 0.06 a	1.27 ± 0.08 a	1.05 ± 0.03 a
8.8	2.25	0	0	0	0
8.8	4.5	0	0	0	0
8.8	9.0	0	0	0	0

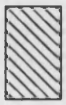
Forty hypocotyl, cotyledon, stem internode and leaf explants of tomato cv. Moneymaker per treatment were cultured on MS medium supplemented with BAP and 2,4-D, incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 µE m^{-2} s^{-1} for 4 weeks.

Values within a column followed by the same letter are not significantly different at 5% according to student's t-test.

Figure 3.9: Morphogenesis of shoot tips explants of tomato cv. Moneymaker cultured on MS medium supplemented with BAP and NAA

Twenty shoot tip explants per treatment were cultured on MS medium supplemented with BAP and NAA, incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1} for 4 weeks.

Key:



Shoot regeneration

Root regeneration

Callus induction

Amount of callus

* Small callus

** Moderate callus

*** Large callus

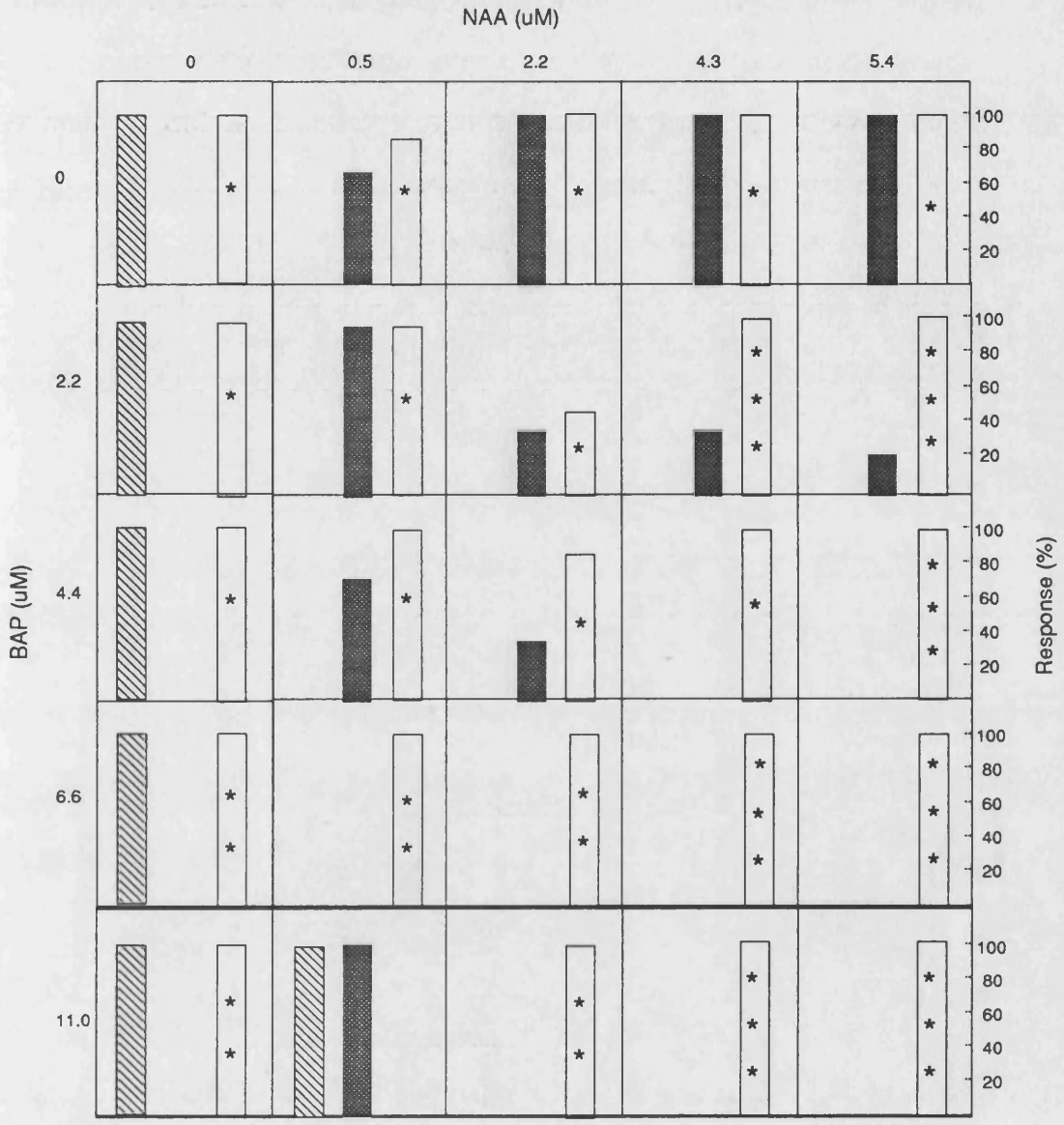


Figure 3.9: Morphogenesis of tomato cv. Moneymaker shoot tip explants on MS supplemented with BAP and NAA

(Figure 3.9). Small amounts of callus were produced on explants cultured on all NAA concentrations, but the amount of callus increased on explants cultured on combinations of NAA with BAP (Figure 3.9).

3.1.4 Effect of Cytokinin and Incubation Period on the Morphogenesis of Hypocotyl Explants of Tomato cv. Moneymaker

This experiment follows the successful production of shoots on tomato explants in response to BAP. Hypocotyl explants of tomato cv. Moneymaker were initiated onto different levels of BAP (0, 8.8, 17.6, 26.4, 35.2, 44.0 μM) and zeatin (0, 9.2, 18.4, 27.6, 36.8, 46 μM). Forty hypocotyl explants were cultured per treatment, incubated in the culture room described in Section 2.3.4 and subcultured every 4 weeks. The morphogenetic response was assessed after 4 weeks and again after 8 weeks.

Shoots were induced after 4 weeks on explants cultured on low concentrations of both cytokinins, but on the higher concentrations shoot regeneration took a longer time (Table 3.3). The frequency of explants with shoot regeneration was higher on explants cultured on BAP compared to the similar level of zeatin and the frequency decreased with increasing concentrations of both cytokinins. The number of responding explants was much higher on BAP than zeatin (Table 3.3). In contrast, the maximum percentage response on zeatin was 52.5% of explants cultured on 18.4 μM. On all BAP media, significantly more shoots ($p < 0.05$) were produced per responding explant in comparison to zeatin (Table 3.3). There was no significant difference in the number of shoots produced per responding explant cultured on the different concentrations of zeatin (Table 3.2). Shoot initiation and development were affected by higher concentrations of both cytokinins, and after 8 weeks small, malformed vegetative structures developed. A large amount of callus was produced

Table 3.3: Effect of cytokinins (BAP and zeatin) and incubation period on the morphogenesis of hypocotyl explants of tomato cv. Moneymaker on MS medium

Plant growth substance (μM)	4 Weeks			8 Weeks	
	Callus		% Explant with shoot initiation	% Explant with shoot initiation	Mean number of shoots > 5 mm per responding explant
	Growth	Colour			
BAP					
0.0	*	Green	30.0	37.6	1.0 d
8.8	***	„	42.5	52.5	1.67 ± 0.10 b
17.6	***	„	67.5	100.0	2.05 ± 0.03 a
26.4	**	White	52.5	80.0	1.46 ± 0.09 c
35.2	*	„	0	44.7	0
44.0	*	„	0	76.5	0
Zeatin					
0.0	*	Green	27.5	32.5	1.0 d
9.2	***	„	37.5	48.6	1.0 d
18.4	**	„	15.0	52.5	1.0 d
27.6	*	White	0	8.3	1.0 d
36.8	*	„	0	52.5	0
46.0	*	„	0	22.5	0

Forty hypocotyl explants of tomato cv. Moneymaker were cultured per treatment (4 explants in a petri dish), incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1}.

Callus growth : * small; ** moderate; *** large.

Values within a column followed by the same letter are not significantly different at 5% according to student's t-test.

on all explants cultured on low levels of both cytokinins, but the amount decreased with increasing concentration of each cytokinin (Table 3.3). No roots were produced on any of the explants cultured on either cytokinin (Table 3.3).

3.1.5 The Response of Hypocotyl Explants of Tomato cv. Moneymaker to a Two-stage Culture System

Friable callus was produced on hypocotyl explants cultured on 4.5 μM 2,4-D (Section 3.1.2.1), and used for organogenesis on different combinations of different plant growth substances. The callus was subcultured to the second stage medium after two weeks of culture initiation. Callus which was induced from a single explant was cultured in a petri dish (5 cm diameter) and considered as a replicate in the following experiments:

a- Kinetin (0, 4.6, and 9.2 μM) and IAA (0, 0.57, 2.85, and 5.7 μM) in a factorial combination with 10 replicates.

b- BAP (0, 8.8, 13.2, 17.6, and 22.0 μM) and IAA (0, 2.28, 2.85 and 3.42 μM) in a factorial combination and each treatment replicated 8 times.

c- BAP (0, 8.8, 13.2, 17.6, and 22.0 μM) and NAA (0, 1.08, 2.7 and 5.4 μM) in a factorial combination with 8 replicates per treatment.

d- BAP (0, 4.4, 8.8, and 13.2 μM) and 2,4-D (0, 0.9, and 2.25 μM) in a factorial combination with 10 replicates per treatment.

e- Zeatin (0, 4.6, 9.2, and 13.8 μM) with 20 replicates per treatment.

f- Zeatin (0, 4.6, and 9.2 μM) and IAA (0, 0.57, 1.14, 2.85 and 5.7 μM) in a factorial combination and with 10 replicates per treatment.

g- Zeatin (0, 4.6, 9.2 and 13.8 μM) and IAA (0, 5.7, 11.4, and 17.1 μM) in a factorial combination with 10 replicates per treatment.

All the above combinations of plant growth substances failed to induce organogenesis. The colour of the callus became brown after 3 weeks of subculture and no new growth developed on the callus until the termination of the experiments after 8 weeks.

The period of callus induction on the first-stage medium (4.5 μM 2,4-D) was reduced to one week before subculturing to the second-stage medium. A small amount of callus was induced on the hypocotyl explants at the time of subculturing. This callus was then subcultured on the combinations of plant growth substances in treatment groups a, b, d and f with eight replicates per treatment and assessed weekly for two months. The reduction of the length of time on the callus inducing media did not affect the organogenesis of the callus of the tomato cv. Moneymaker. Explants became brown after 4 weeks and no new growth of callus was produced in any treatment.

3.1.6 Origin of Adventitious Shoots on Hypocotyl Explants

It was not clear from observations whether adventitious shoots on hypocotyl explants of Moneymaker seedlings originated from callus, or directly from the hypocotyl itself. A preliminary investigation was carried out to identify the origin of these shoots. Hypocotyl explants cultured on MS medium containing 8.8 μM BAP were harvested after 8 days, and subsequently every 2 days until day 24. Four explants from each date were then dehydrated, infiltrated, embedded and sectioned as described in Section 2.4. Callus had begun to form at the cut ends of the hypocotyl by day eight. After 16 days organised meristematic regions could be clearly identified at the periphery of the callus. There were no apparent vascular connections between the meristematic regions and the regional hypocotyl tissue (Plates 3a-3c).

PLate 3.3a-c: Sectioning of tomato cv. Moneymaker hypocotyl explants:

Plate 3.3a Callus induced by 4.4 μ M BAP 14 days after cultures initiation.

Note the Xylem tissue (X) on the callus.

Bar = 200 μ

Plate 3.3b Showing the meristematic tissue on the callus periphery 16 days after culture initiation.

Scattered xylem tissue and no continuity between the meristimatic tissue and the original explant

Bar = 100 μ

Plate 3.3c Magnifications of the meristematic tissue

Note the organized layers of large nucleated cells

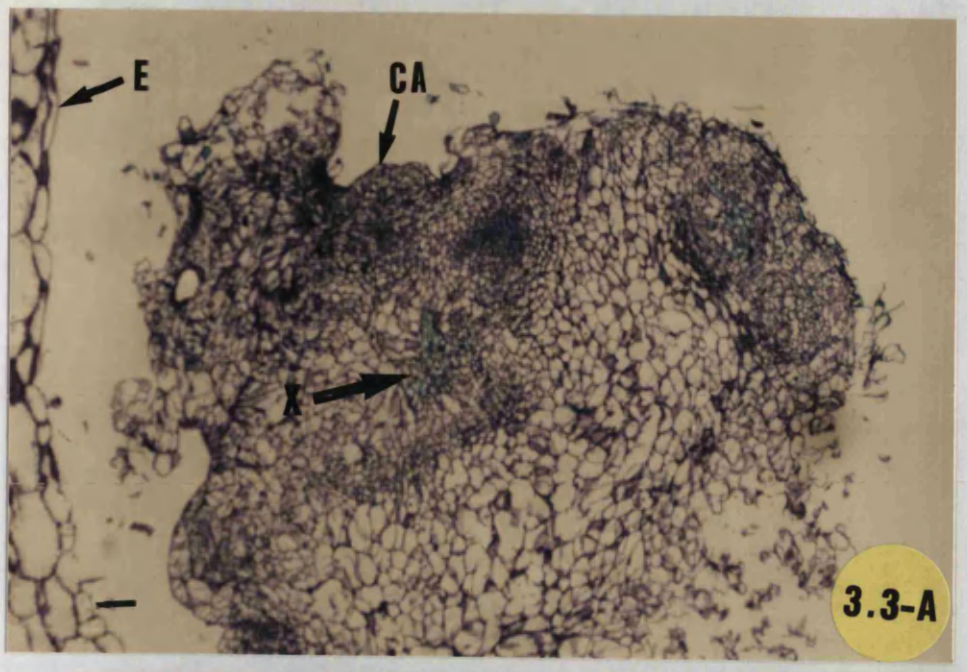
Bar = 40 μ

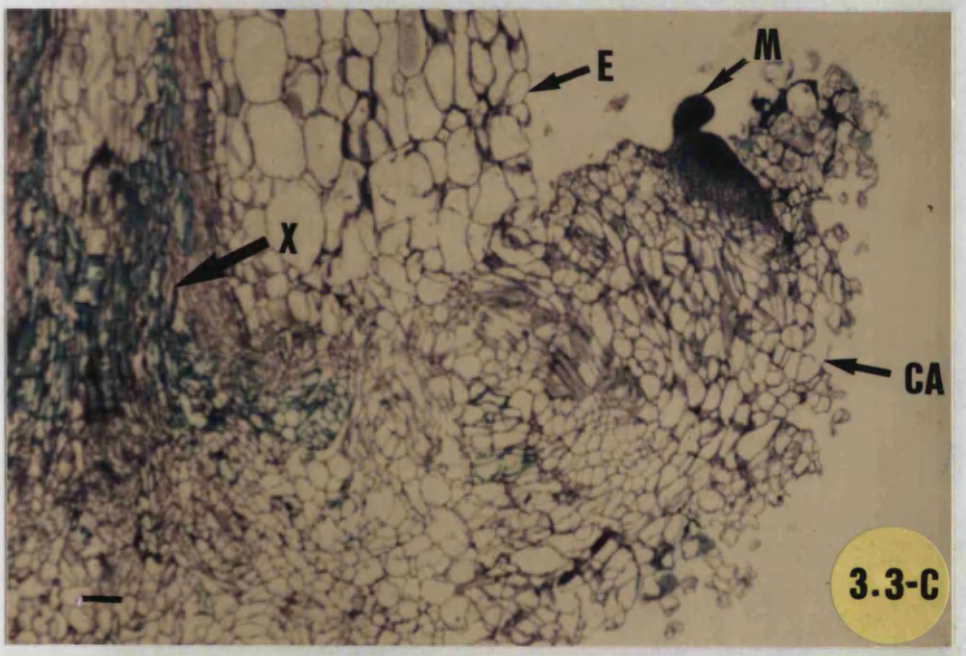
C = Callus

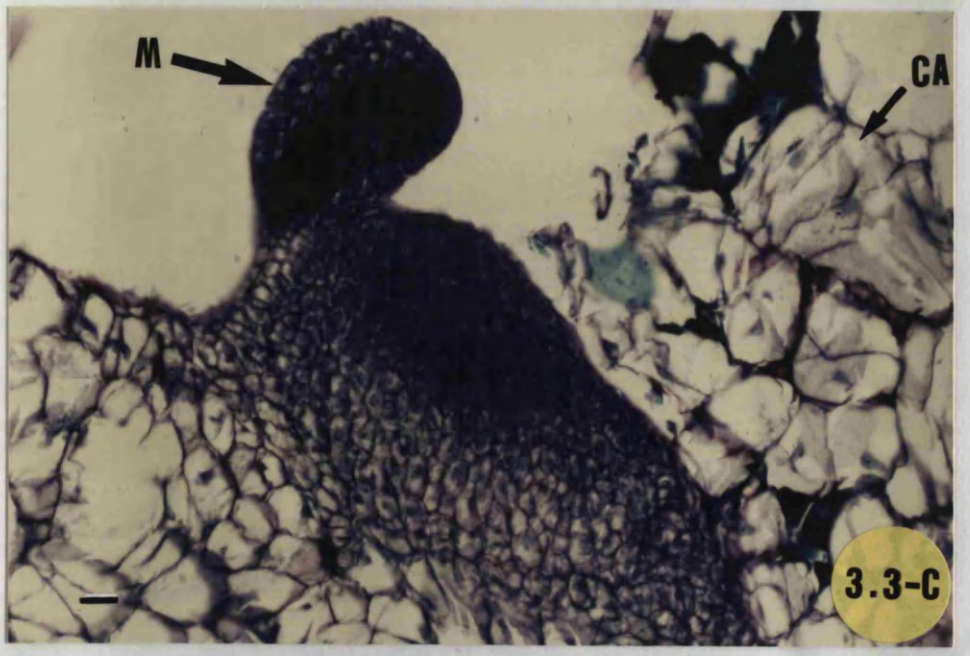
E = original explant

X = Xylem

M = Meristematic tissue


E
CA
X
3.3-A


E
M
X
CA
3.3-C


M
CA
3.3-C

3.1.7 Rooting of Tomato Plants Regenerated from cv. Moneymaker on Liquid MS Containing NAA

Initially, a small range of auxin media were tested for rooting of tomato regenerants, 0, 2.25, 4.5 and 9.0 μM NAA. Twenty shoots, which were regenerated from hypocotyl explants cultured on MS containing 8.8 μM BAP, were tested in each treatment. The plants were rooted on a filter paper bridge in 10.0 ml liquid MS medium containing NAA. The test tubes were then incubated in the culture room described in Section 2.3.4 for two weeks before roots were observed. A high frequency of plants rooted on all concentrations of NAA as well as on the auxin free medium (Table 3.4). The high concentration of NAA produced few and long roots without root hairs, but a dense root system was produced on the explants cultured on the lower concentrations of NAA (Table 3.4). Roots on explants cultured on MS without auxin resembled those produced on the high level of NAA, but they had fewer root hairs (Table 3.4).

Table 3.4: Rooting of tomato cv. Moneymaker somaclones on liquid MS medium containing NAA

NAA (μM)	% Rooted Plants	Description of roots
0.0	85	2-5 long roots/plant with few hairs.
2.25	95	Dense root system with few root hairs.
4.5	95	Dense root system with dense root hair.
9.0	95	6-10 long roots/plant without root hairs.

Twenty somaclones regenerated from hypocotyl explants of tomato cv. Moneymaker were cultured on filter paper bridges in 30 ml test tubes, and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1}.

3.1.8 Morphogenesis of Tomato cv. Rootstock Explants cultured on MS Medium Supplemented with BAP and NAA or 2,4-D

Seeds of tomato cv. Rootstock were sterilized and germinated *in vitro* as described in Section 2.3. Hypocotyl, cotyledon, leaf and stem internode explants from 4 week-old seedlings were cultured on MS medium containing combinations of BAP and NAA (0, 0.1, 1.0, and 10 μM each). Forty explants per treatment were incubated in the culture room described in Section 2.3.4 for four weeks before the morphogenetic response was assessed. In a second experiment, the same levels of BAP were used but 2,4-D was used instead of NAA.

3.1.8.1 The Morphogenetic Effect of BAP and NAA on Explants of Tomato cv. Rootstock

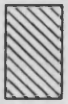
Cytokinin induced shoot regeneration on hypocotyl explants and the number of responding explants increased with the increasing BAP concentration up to 1.0 μM and then decreased (Figure 3.10 and Plate 3.4). The combination of 10 μM BAP with 0.1 μM NAA also produced shoots on 28% of the hypocotyl explants, but other combinations of BAP and NAA failed to induce shoot regeneration on the hypocotyl explants. Also the hypocotyl explants cultured on NAA alone failed to regenerate shoots, but shoots were produced on some explants cultured on MS without plant growth substances (Figure 3.10). The number of shoots produced per responding hypocotyl explant was significantly higher ($p < 0.05$) on all BAP levels compared to the number of shoot produced on MS containing no plant growth substances or the shoots regenerated on a combination of BAP and NAA (Table 3.5).

Hypocotyl explants regenerated roots when cultured on NAA media as well as on the combinations of NAA and low concentrations of BAP (Figure 3.10). The

Figure 3.10 and 3.11 : Morphogenesis of hypocotyl and cotyledon explants of tomato cv. Rootstock cultured on MS medium supplemented with BAP and NAA

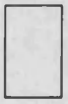
Forty explants per treatment were cultured on MS medium supplemented with BAP and NAA, and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1} for 4 weeks.

Key:



Shoot regeneration

Root regeneration



Callus induction

Amount of callus

* Small callus

** Moderate callus

*** Large callus

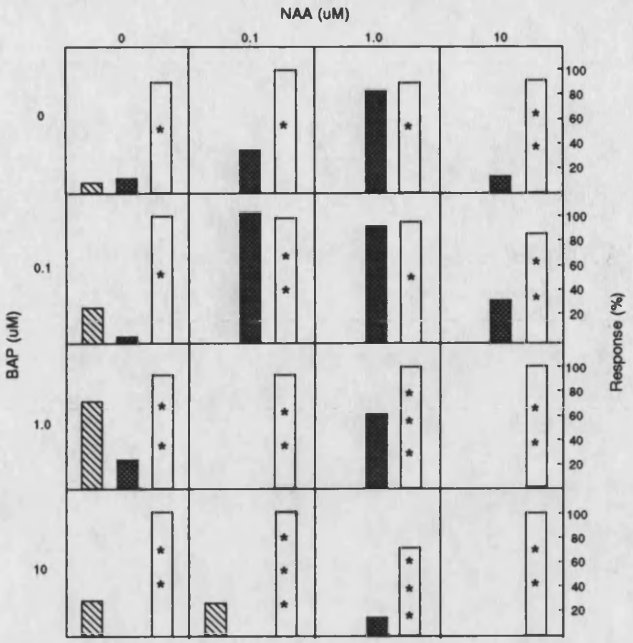


Figure 3.10 : Morphogenesis of tomato cv. Rootstock hypocotyl explants on MS medium supplemented with BAP and NAA

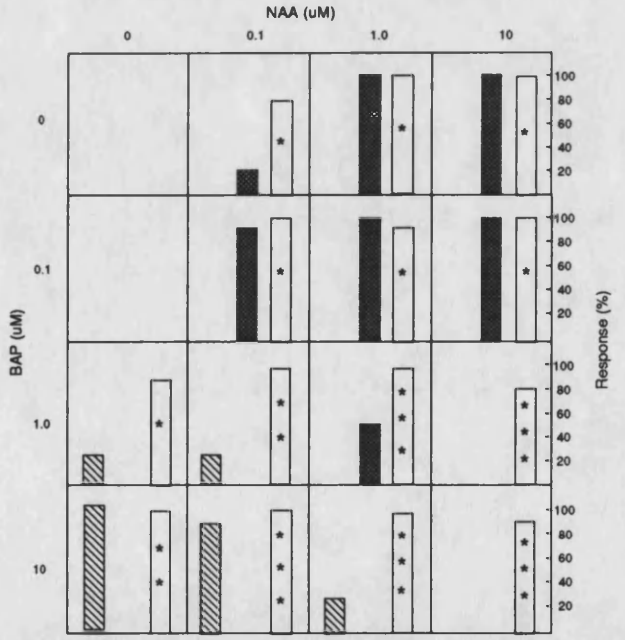
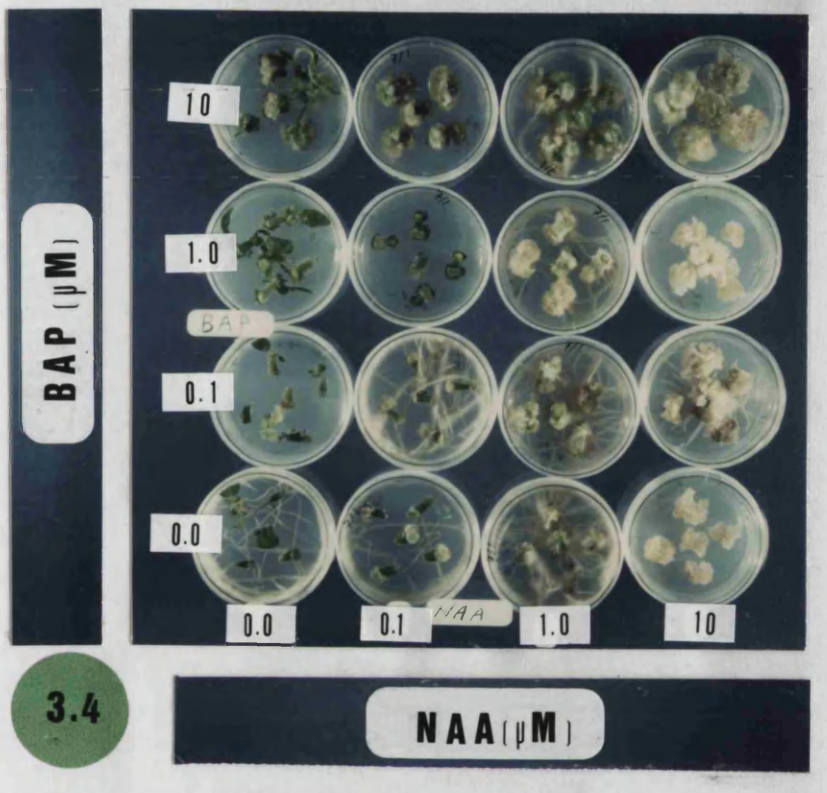


Figure 3.11 : Morphogenesis of tomato cv. Rootstock cotyledon explants on MS medium supplemented with BAP and NAA

Plate 3.4: Morphogenesis of tomato cv. Rootstock hypocotyl explants on MS supplemented with BAP and NAA 4 weeks after culture initiation



3.4

combination of 1.0 μM NAA and the high concentrations of BAP also induced root regeneration, but other levels of NAA failed to do so when combined with high levels of BAP (Figure 3.10). A small number of hypocotyl explants regenerated roots on MS containing low levels of BAP, But more were produced on the highest BAP concentration (Figure 3.10).

All combinations of BAP and NAA induced callus production on a high percentage of hypocotyl explants (Figure 3.10). The amount of callus increased when the concentration of BAP or NAA was increased from 0 to 10 μM (Figure 3.10). The amount of callus produced was small to moderate when hypocotyl explants were cultured on NAA, BAP and the combination of NAA with low BAP concentrations, but the callus size increased when high concentrations of both plant growth substances were combined (Figure 3.10).

Shoots were regenerated on cotyledon explants cultured on high concentrations of BAP as well as the combination of these levels and 0.1 μM NAA (Figure 3.11). The combination of 10 μM BAP and 1.0 μM NAA also caused shoot regeneration on 27% of the cotyledon explants, but low concentrations of BAP and the remaining combinations of BAP and NAA did not induce shoot regeneration on cotyledon explants (Figure 3.11). The number of shoots produced per explant on BAP media was significantly higher in comparison with that produced on BAP and NAA combination (Table 3.5). No difference was found between BAP concentrations in the number of shoot produced per explant. Roots were induced on cotyledon explants by NAA alone, and NAA in combination with 0.1 μM BAP. No roots were produced on cotyledon explants cultured on NAA when combined with high levels of BAP.

A clear pattern of morphogenesis was apparent from Rootstock leaf explants cultured on different combinations of BAP and NAA (Figure 3.12). No shoots were produced on the lower concentrations of BAP or the highest concentration of NAA.

Table 3.5: Number of shoots per responding explant of tomato cv. Rootstock on MS supplemented with BAP and NAA

Plant growth substance µM		Mean number of shoots per responding Explant		
BAP	NAA	Hypocotyl	Cotyledon	Leaves
0	0	1.0 b	0	0
0	0.1	0	0	0
0	1.0	0	0	0
0	10	0	0	0
0.1	0	1.57 ± 0.12 a	0	0
0.1	0.1	0	0	0
0.1	1.0	0	0	0
0.1	10	0	0	0
1.0	0	1.38 ± 0.13 a	1.31 ± 0.15 a	1.40 ± 0.11 a
1.0	0.1	0	1.0 b	1.0 b
1.0	1.0	0	0	0
1.0	10	0	0	0
10	0	1.55 ± 0.23 a	1.56 ± 0.12 a	1.40 ± 0.08 a
10	0.1	1.0 b	1.0 b	1.0 b
10	1.0	0	0	1.0 b
10	10	0	0	0

Values within a column followed by the same letter are not significantly different at 5% according to student's t-test.

Figure 3.12: Morphogenesis of leaf explants of tomato cv. Rootstock cultured on MS medium supplemented with BAP and NAA

Figure 3.13: Morphogenesis of hypocotyl explants of tomato cv. Rootstock cultured on MS medium supplemented with BAP and 2,4-D

Forty explants per treatment were cultured on MS medium supplemented with plant growth substance, and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 μE m^{-2} s^{-1} for 4 weeks.

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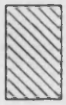
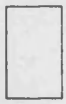


Shoot regeneration

Root regeneration



Callus induction

Amount of callus

* Small callus

** Moderate callus

*** Large callus

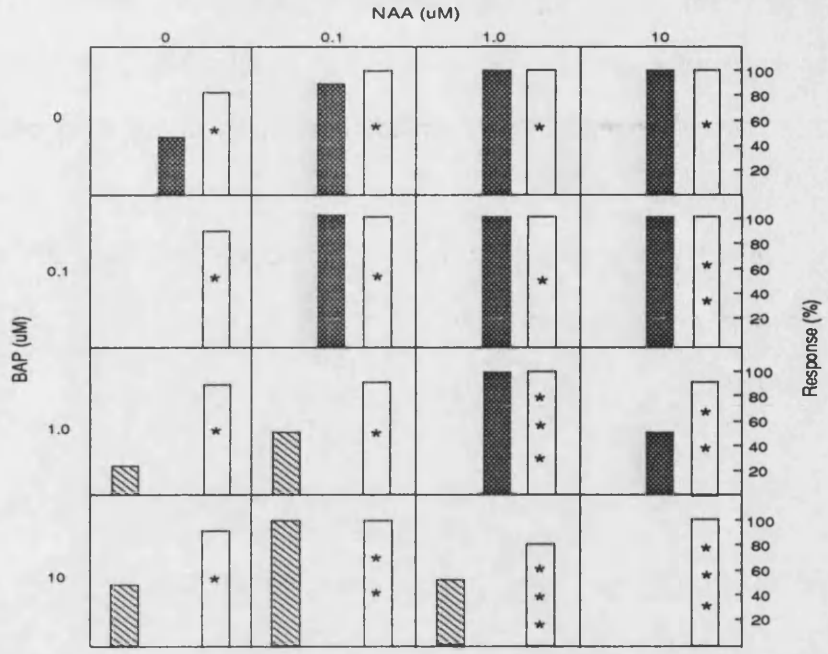


Figure 3.12 : Morphogenesis of tomato cv. Rootstock leaf explants on MS medium supplemented with BAP and NAA

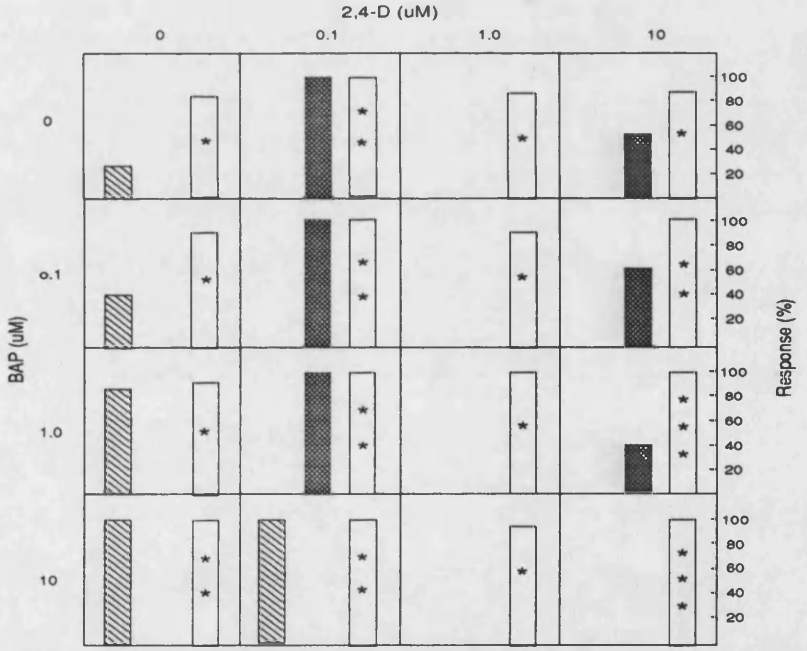


Figure 3.13 : Morphogenesis of tomato cv. Rootstock hypocotyl explants on MS supplemented with BAP and 2,4-D

Twenty four and 44% of explants produced shoots on 1.0 μM and 10 μM BAP respectively. The percentage of responding explants was further increased by the addition of NAA to the media, up to 100% on media containing 10 μM BAP plus 0.1 μM NAA (Figure 3.12). Shoot regeneration was induced on high concentrations of BAP alone and the combination of these BAP levels with low NAA concentrations. The number of shoots produced per leaf explant was higher on BAP media in comparison with BAP/NAA combinations (Table 3.5). The number of shoots produced per explant was similar at different BAP concentrations. Most leaf explants produced roots on media containing low or no BAP. Callus was produced at the cut surface of a high percentage of explants in all treatments. The actual amounts of callus increased with the higher concentrations of combined plant growth substances (Figure 3.12).

3.1.8.2 The Morphogenetic Effect of BAP and 2,4-D on Explants of Tomato cv. Rootstock

The percentage of hypocotyl explants producing shoots increased with increasing concentration of cytokinins in the media (Figure 3.13 and Plate 3.5), from 25% on 0 BAP to 100% on 10 μM BAP. Shoots were also initiated on explants cultured on the highest concentrations of BAP with 0.1 μM 2,4-D. Higher levels of 2,4-D however inhibited shoot regeneration. No shoots were produced on any other medium which included 2,4-D. Mainly single shoots were produced on low concentrations of BAP, and the number of shoots produced per responding hypocotyl explant increased on higher BAP levels. The number of shoots produced per explant was significantly higher on all BAP concentrations in comparison with the control and the combination of the two plant growth substances (Table 3.6). No difference was found between the number of shoots regenerated on the different concentrations of BAP (Table 3.6).

Plate 3.5: Morphogenesis of tomato cv. Rootstock hypocotyl explants on MS supplemented with BAP and 2,4-D

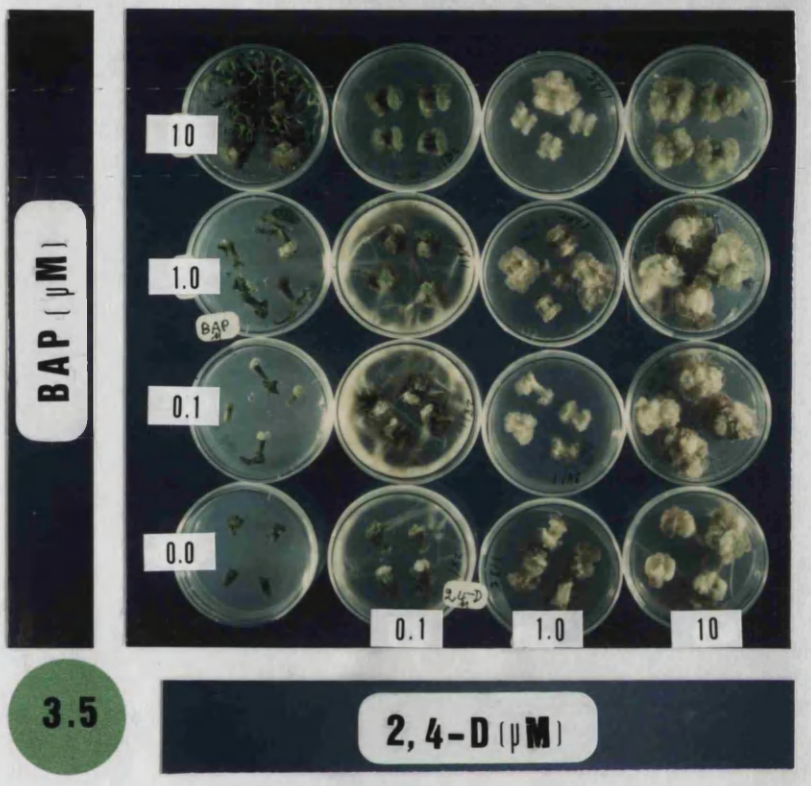

BAP (μM)
10
1.0
0.1
0.0
0.1
1.0
10
3.5
2, 4-D (μM)

Table 3.6 Number of shoots per responding explant of tomato cv. Rootstock on MS supplemented with BAP and 2,4-D

Plant growth substance µM		Mean number of shoots per responding Explant		
BAP	2,4-D	Hypocotyl	Cotyledon	Leaves
0	0	1.0 b	0	0
0	0.1	0	0	0
0	1.0	0	0	0
0	10	0	0	0
0.1	0	1.20 ± 0.07 a	0	0
0.1	0.1	0	0	0
0.1	1.0	0	0	0
0.1	10	0	0	0
1.0	0	1.81 ± 0.13 a	1.31 ± 0.08 a	1.40 ± 0.09 a
1.0	0.1	0	0	0
1.0	1.0	0	0	0
1.0	10	0	0	0
10	0	1.76 ± 0.13 a	1.84 ± 0.13 a	1.57 ± 0.27 a
10	0.1	1.0 b	1.0 b	1.0 b
10	1.0	0	0	0
10	10	0	0	0

Values within a column followed by the same letter are not significantly different at 5% according to student's t-test.

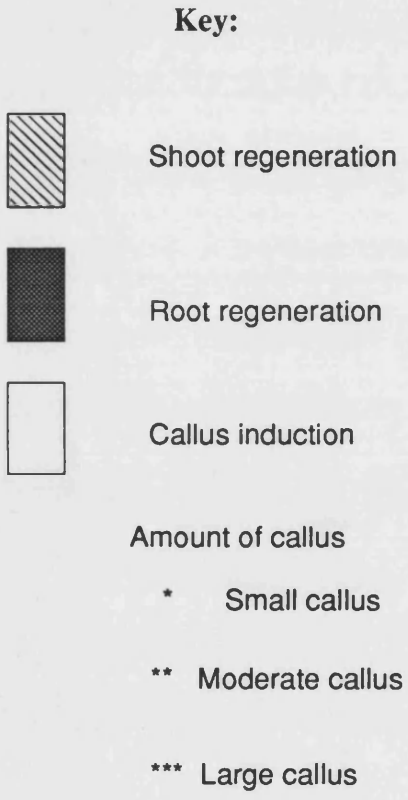
Roots were regenerated on 0.1 μM and 1.0 μM 2,4-D with or without low concentrations of BAP (Figure 3.13). No roots were produced on the other media. Callus was produced at the cut surface of a high percentage of explants cultured on all combinations of both plant growth substances. The amount of callus produced on low levels of BAP and 2,4-D was small, but increased on the media containing high concentrations of these plant growth substances (Figure 3.13).

The cotyledon and leaf explants of Rootstock showed a similar pattern of morphogenesis on BAP and 2,4-D media (Figures 3.14 and 3.15). Shoots were produced on cotyledon and leaf explants cultured on 1.0 μM BAP alone or combined with 0.1 μM 2,4-D, but no shoots were regenerated on the remaining combinations of these plant growth substances (Figures 3.14 and 3.15). The number of shoots regenerated per responding leaf and cotyledon explant was significantly higher ($p < 0.05$) on high BAP concentrations compared to that produced on the combination of BAP and 2,4-D (Table 3.6). The number of shoots produced per explant was not significantly different between BAP concentrations on either explant (Table 3.6).

Friable callus was produced at the cut surface of both cotyledon and leaf explants cultured on all combinations of 2,4-D and BAP (Figures 3.14 and 3.15). Large amounts of callus were produced when these explants were cultured on a combination of high concentrations of both plant growth substances, whilst most of the remaining combinations produced small amounts of callus (Figures 3.14 and 3.15). Roots were produced on leaf and cotyledon explants cultured on 0.1 μM and 10 μM 2,4-D, and the combinations of these 2,4-D concentrations with low concentration of BAP (Figures 3.14 and 3.15). In addition, the combinations of 10 μM of either plant growth substance with 1.0 μM of the other produced roots on leaf explants (Figure 3.15).

Figure 3.14 and 3.15: Morphogenesis of cotyledon and leaf explants of tomato cv. Rootstock cultured on MS medium supplemented with BAP and 2,4-D

Forty explants per treatment were cultured on MS medium supplemented with BAP and 2,4-D and incubated at 25 ± 1 °C, 16 hours photoperiod and light intensity of 50 $\mu E\ m^{-2}\ s^{-1}$ for 4 weeks.



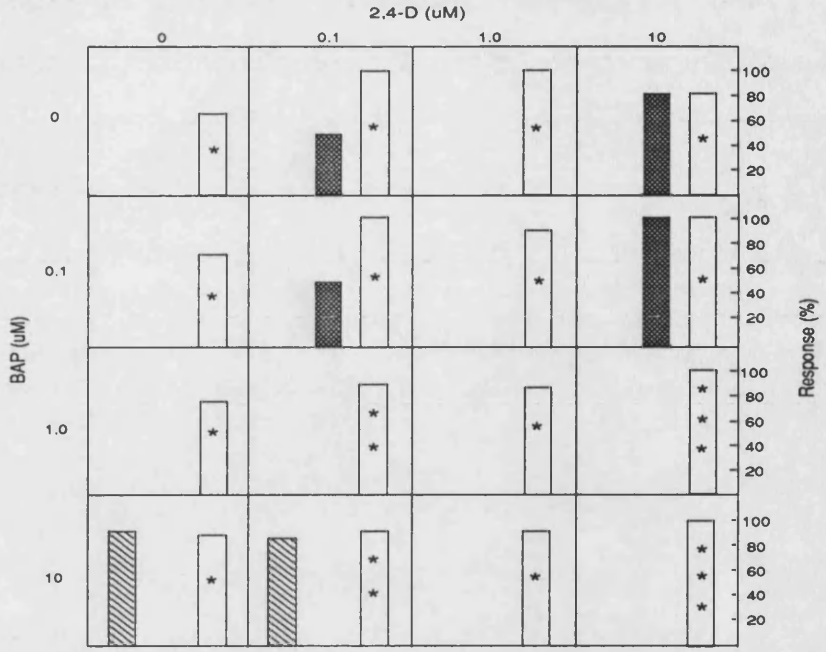


Figure 3.14 : Morphogenesis of tomato cv. Rootstock cotyledon explants on MS medium supplemented with BAP and 2,4-D

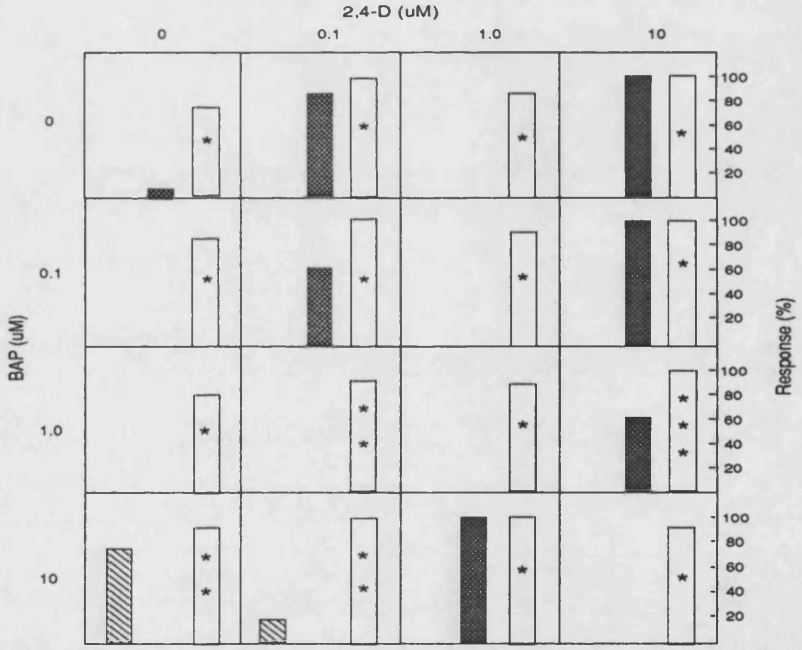


Figure 3.15 : Morphogenesis of tomato cv. Rootstock leaf explants on MS supplemented with BAP and 2,4-D

3.1.9 The Morphogenetic Effect of Cytokinins on Hypocotyl Explants of Tomato cv. Rootstock Cultured on B5 Medium

The hypocotyl explants of 4 weeks-old seedlings of tomato cv. Rootstock were cultured on B5 medium containing BAP (0, 4.4, 17.6, and 26.4 μM) or kinetin (0, 9.4, 18.8, 28.2 μM). Twenty eight explants per treatment were incubated in a culture room, described in Section 2.3.4, for 4 weeks before the morphogenetic response was assessed and presented in Table 3.7. Both cytokinins stimulated shoot initiation on the hypocotyl explants of cv. Rootstock, and the percentage of responding explants varied with the cytokinin and its concentration (Table 3.7). On kinetin media the highest percentage of explants producing shoots was found on 9.4 μM. The number of shoots per responding explant increased with increasing concentration of kinetin, and the number of shoots produced by kinetin at all concentrations was significantly higher ($p < 0.05$) than the ones produced on B5 with no plant growth substances (Table 3.7). BAP at 17.4 μM produced shoots on the highest percentage of explants followed by 8.8 μM. The number of shoots produced per explant on all BAP concentrations were not significantly different, but they were significantly higher ($p < 0.05$) compared with those produced on B5 medium free of plant growth substance (Table 3.7). However, the number of shoots produced per responding explant was similar on both cytokinins at all concentrations (Table 3.7). Roots were regenerated on explants cultured on B5 with no plant growth substances (Table 3.7). Large and friable callus was produced on all kinetin and BAP concentrations except 18.8 μM BAP, on which callus of moderate size was produced (Table 3.7). Little callus was produced on B5 medium.

Table 3.7: Effect of cytokinins (BAP and Kinetin) on the morphogenesis of hypocotyl explants of tomato cv. Rootstock cultured on B5 medium

Cytokinin (µM)	Callus growth	% Explant with roots	% Explant with shoots	Mean number of Shoots per responding explant
B5	*	66.7	7.1	1.0 b
9.2 Kinetin	***	0	78.6	2.22 ± 0.22 a
18.8 Kinetin	***	0	60.7	2.54 ± 0.53 a
28.2 Kinetin	***	0	53.6	3.13 ± 0.56 a
8.8 BAP	***	0	57.1	2.75 ± 0.30 a
17.6 BAP	***	0	71.4	2.40 ± 0.14 a
26.4 BAP	**	0	17.9	2.20 ± 0.33 a

Twenty eight explants per treatment.

Callus growth : * small ; ** moderate ; *** large.

Values within a column followed by the same letter are not significantly different at 5% according to student's t-test.

3.1.10 Variability between Tomato Somaclones

Plants used for variability tests were regenerated from hypocotyl explants of both tomato cultivars (cvs. Moneymaker and Rootstock). All somaclones were cloned *in vitro* and rooted on 5.4 μM NAA which produced a dense root system with dense root hairs on cv Moneymaker (Section 3.1.8). These plants were transplanted to F2 compost and placed under a misting unit for a week in a greenhouse at 20 °C minimum temperature. Plants were assessed for genetic and morphological variation 3 weeks after the transplanting into F2 compost.

3.1.10.1 Chromosome Number in Somaclones of Tomato cv. Moneymaker

Healthy and actively growing root tips were selected from each somaclone and incubated for four hours at 17-20 °C in 8-hydroxyquinoline (350 mg l^{-1}). The root tips were then transferred to a 1:3 acetic acid/ethanol (95%) mixture, in which they were stored for at least 24 hours before maceration in 1 M HCl for 5 minutes at 60 °C. The chromosomes were stained and squashed in lactopropionic orcein. The ploidy level of the regenerated tomato plants was determined by counting the chromosomes in five cells per root tip under the light microscope.

The chromosome numbers in 11.9% of 42 plants regenerated from tomato cv. Moneymaker was 48 (tetraploid), but the remaining regenerants were diploid, with 24 chromosomes (Plates 3.6a-c). The polyploid regenerants showed different morphology compared to the diploid ones; dark green colour, broader leaves and no lateral branches (Plate 3.7). In addition, excessive flower shedding and low fertility was found with the tetraploid plants. Seeds were collected from the fertile somaclones after selfing.

Plate 3.6: Variability in number of chromosomes in the root tip of tomato plants regenerated from Moneymaker 3 weeks after transplanting.

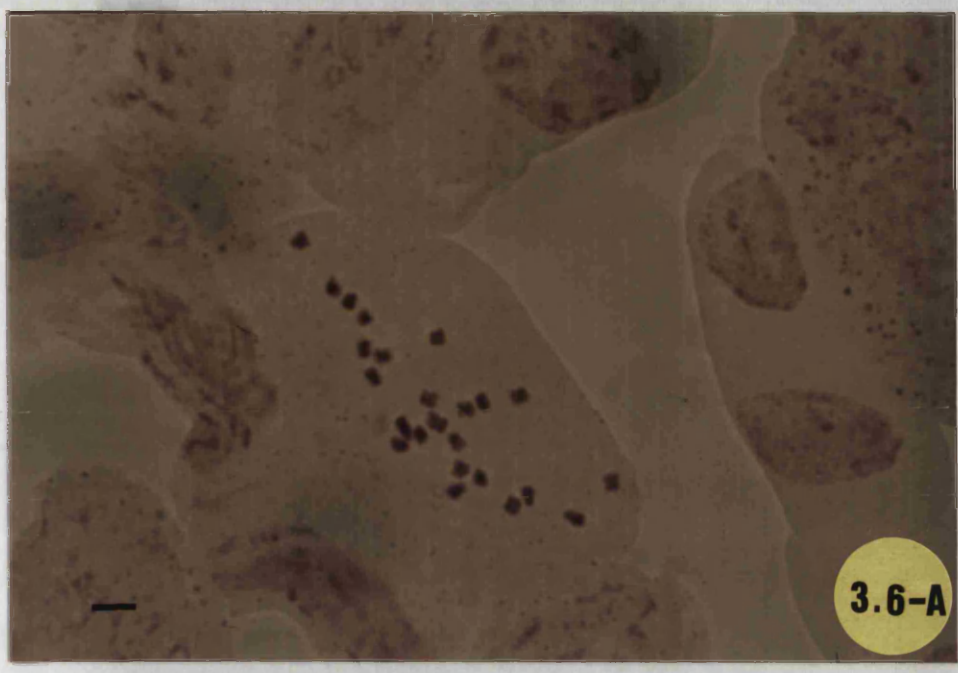
Plate 3.6a: Diploid plant with 24 chromosomes (same as Moneymaker)

Plate 3.6b: Tetraploid plant with 48 chromosomes

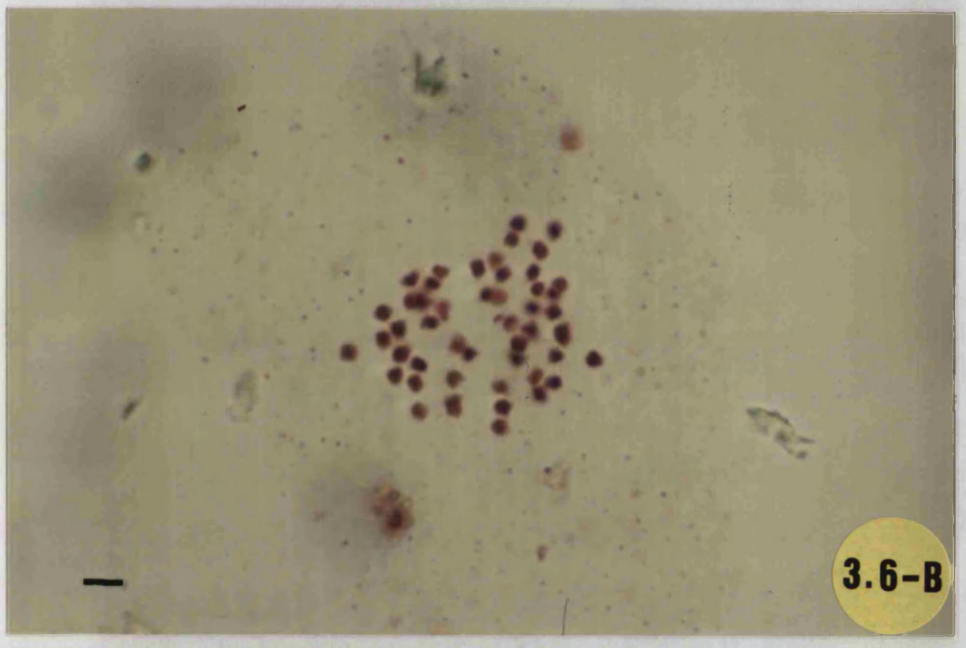
Bar = 5 μ

Plate 3.7: The variability in leaf and plant morphology between tomato plants regenerated from cv. Moneymaker (A).

The tomato somaclones (C and B) with wide dark green leaves compared to cv. Moneymaker.



3.6-A



3.6-B

3.7

3.1.10.2 Ploidy Level of Tomato Somaclones Regenerated from cv. Rootstock

The ploidy levels of tomato plants regenerated from cv. Rootstock were assessed by the number of chloroplasts per pair of guard cells on epidermal pieces from the lower leaf surface which is well established on tomato (Koornneef et al., 1989; Bulk et al., 1990). This simple method was used instead of the lengthy one used for chromosome counting in Section 3.1.10.1, to classify the regenerants into 2 groups (diploid or polyploid). Leaflets for this study were collected from the terminal leaflet of the third leaf of each plant to avoid the abnormalities on the first leaf caused by the culture conditions. The epidermal pieces were then placed on slides with a film of water and examined under blue light with a fluorescent microscope (Section 2.6.4). Chloroplasts were counted in 20 pairs of guard cells replicated 3 times per plant. The results of 120 somaclones showed that 17.5% were polyploids and the remainder were diploids (Figure 3.16). These polyploids had higher chloroplast numbers per guard cell compared to that of cv. Rootstock (Plates 3.8a and b). The polyploidy was mainly tetraploid, confirmed by the number of chromosomes counted in some of the regenerants. Only one plant was found with 34 chromosomes. Three polyploid plants with stunted growth (Plate 3.9), and others with morphological variations such as size and shape of the leaflet which became wider or smaller, in addition to the change in the number of leaflets per leaf were found (Plate 3.10).

3.1.10.3 Variation between the Progenies of Moneymaker Somaclones

The tetraploid regenerants of tomato cv. Moneymaker did not produce seeds in the greenhouse conditions (Section 2.2) and seeds were only collected from the diploid somaclones. Five seeds from each line were germinated and tested for genetic and morphological variability 4 weeks after germination. The length of time needed for flower initiation was also recorded and the chloroplast numbers per guard cell

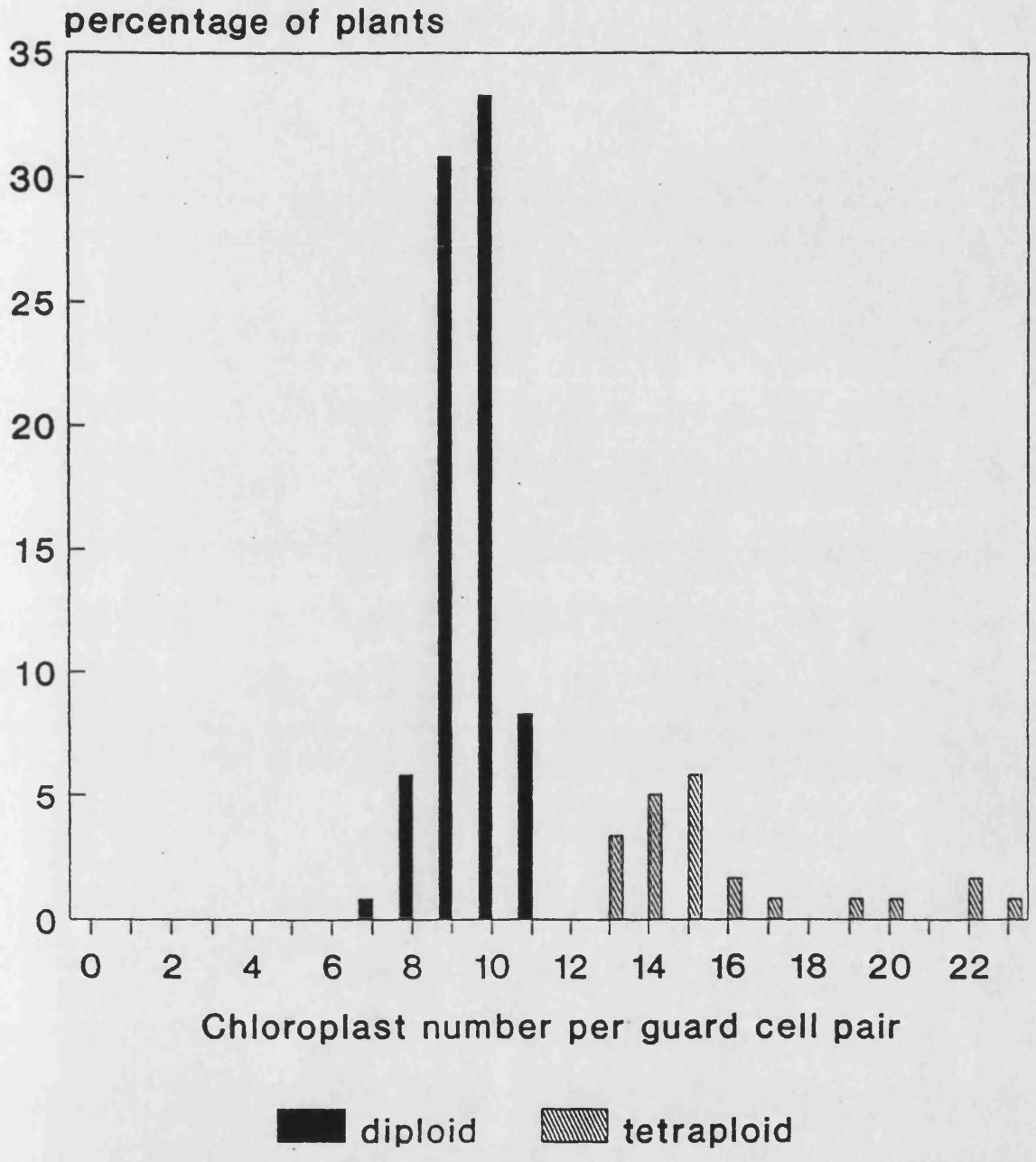


Figure 3.16: Frequency distribution of chloroplasts number counted per guard cell pair of diploid and polyploid tomato somaclones regenerated from cv. Rootstock.

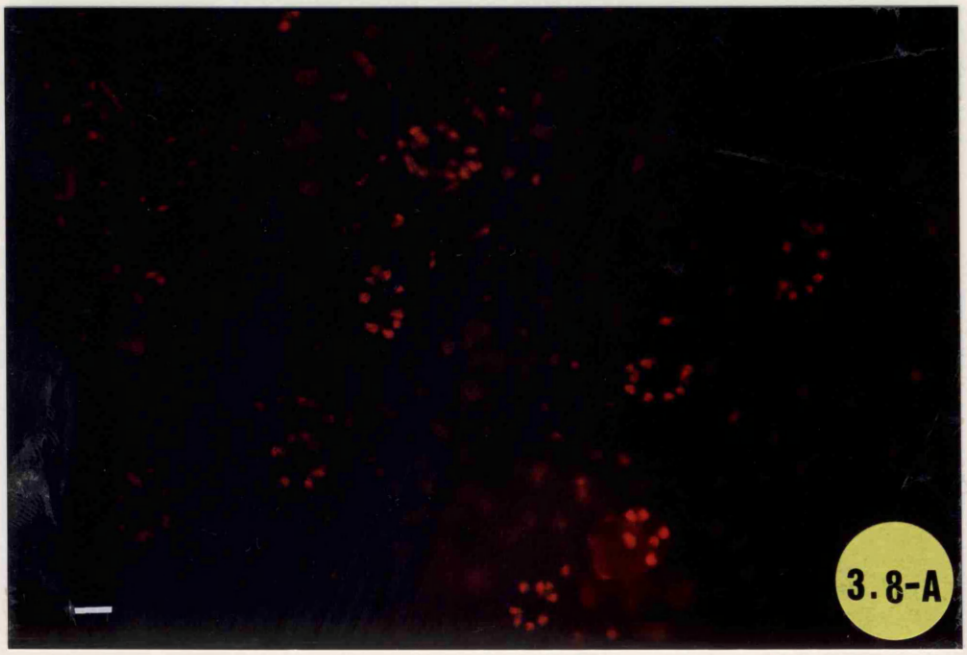
Chloroplasts were counted in 20 pairs of guard cells, on 3 epidermal pieces per leaflet, under a fluorescence microscope with blue light and 530 nm barrier filter. The number of chromosomes in root tips of 2-3 plants selected from each group were also counted for conformation.

Plate 3.8: Variability in number of chloroplasts per pair of guard cells on epidermal strips of tomato plants regenerated from cv. Rootstock

Plate 3.8a: Represents diploid regenerant of tomato (same as Moneymaker)

Plate 3.8b: Represents tetraploid regenerant of tomato

Bar = 100 μ


3.8-A

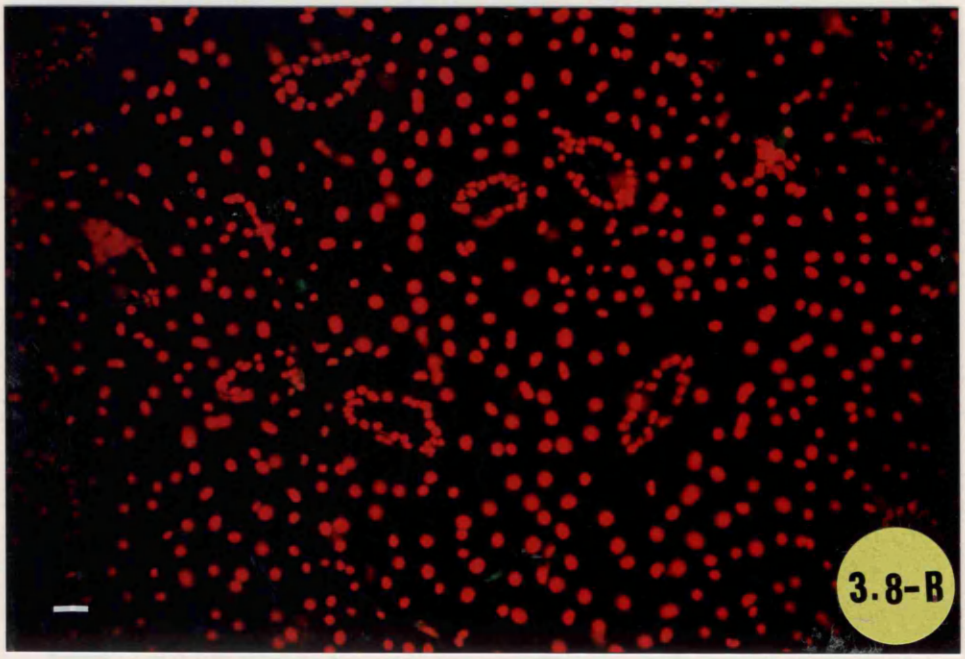

3.8-B

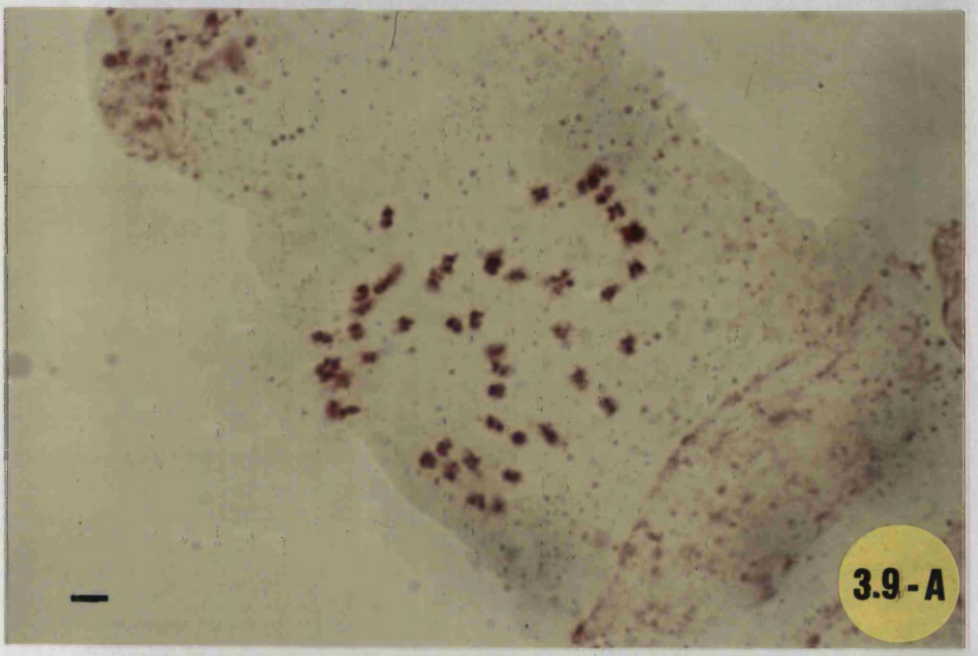
Plate 3.9a: Chromosome numbers (34) in Rootstock somaclones

Bar = 5 μ

Plate 3.9b: Variability of growth habit between tomato plants regenerated from cv. Rootstock. Note the stunted growth of the somaclone (A)

Plate 3.10: Differences in leaf shape between tomato cv. Rootstock regenerants Differences in the no.of leaflets per leaf, arrangement and leaflet width.

c = control (Rootstock)


3.9 - A


A
B
3.9 - B


C
3.10

pair was counted on the terminal leaflet of the first leaf (3 epidermis pieces per leaflet). The number of stomata in a microscopic (mean of 4 fields, 1.0 mm diameter each) and the leaf ratio (length /width) of 9 leaflets per plant (3 leaflets were taken from each leaf) were assessed. No differences in the ploidy level between these lines was found as the number of chloroplasts (Table 3.8) showed a range from 8.03 to 11.40, and these progenies were diploid plants as confirmed by the number of chromosomes in the root tips of 3 plants from each group. Some of the progenies had wider leaflets than Moneymaker, and the initiation of flowering of these plants varied from 38 to 44 days (Table 3.8).

3.2 Discussion

Shoot regeneration systems from cultures of different types of tomato explants were established. One-stage culture on BAP alone was effective in regenerating shoots from hypocotyl, cotyledon, leaf and stem internode explants of two tomato cultivars, Moneymaker and Rootstock (Sections 3.1.1 and 3.1.8). These results are in agreement with others (Kartha et al., 1976, 1977; Novak and Maskova, 1979; Compton and Veilleux, 1991; Gunay and Rao, 1980; Di Paola et al., 1983), who reported shoot regeneration from different explants of tomato using cytokinins alone, (BAP and zeatin). Novak and Maskova (1979) reported that all the levels of BAP and kinetin from 1.0 to 10 μM induced shoot regeneration from the apical meristem of tomato cv. Moneymaker and multiple shoot regeneration was induced on the concentrations ≥ 5 μM BAP. A similar effect of BAP on the apical meristem of tomato cv. Starfire was reported by Kartha et al. (1977). After investigating the regeneration potential of leaf explants of 8 tomato species and two related *Solanum* species, Kut and Evans (1984) concluded that 5 μM BAP was sufficient for regeneration of most of these species.

Table 3.8: Evaluation of the variability between the progenies of tomato somaclones regenerated from Moneymaker

Progenies of Tomato Somaclones (SC_2)	Mean No. of Chloroplasts per Guard Cell Pair	Mean No. of Stomata per 1.0 mm dia.' field	Mean Leaf Ratio	Mean No. of Days to Flowering
1	9.6 ± 0.52	72.8 ± 2.72	2.3 ± 0.12	41.9 ± 0.13
2	9.6 ± 0.12	48.5 ± 2.02	2.1 ± 0.14	40.2 ± 0.16
4	9.3 ± 0.19	68.8 ± 3.17	2.2 ± 0.04	44.4 ± 0.26
5	8.8 ± 0.18	53.5 ± 3.28	2.3 ± 0.09	39.8 ± 0.53
6	9.5 ± 0.21	50.0 ± 1.47	2.2 ± 0.07	44.1 ± 0.13
8	8.2 ± 0.14	117.3 ± 6.80	2.4 ± 0.02	39.0 ± 0.38
9	11.1 ± 0.35	58.5 ± 4.43	2.2 ± 0.08	39.3 ± 0.16
10	9.0 ± 0.31	51.0 ± 1.08	2.3 ± 0.08	43.1 ± 0.48
11	9.2 ± 0.25	71.5 ± 6.05	2.3 ± 0.03	40.1 ± 0.13
12	10.4 ± 0.36	51.0 ± 3.72	2.2 ± 0.06	42.5 ± 0.38
15	10.0 ± 0.68	78.0 ± 4.67	2.3 ± 0.07	39.6 ± 0.32
16	8.0 ± 0.14	60.0 ± 4.53	2.3 ± 0.06	39.1 ± 0.13
17	9.6 ± 0.59	65.5 ± 5.20	2.4 ± 0.07	42.3 ± 0.16
18	9.2 ± 0.48	54.3 ± 4.30	2.2 ± 0.05	43.8 ± 0.53
20	8.0 ± 0.14	48.8 ± 2.43	2.3 ± 0.07	43.5 ± 0.50
21	8.5 ± 0.25	50.5 ± 1.75	2.3 ± 0.08	43.3 ± 0.16
22	9.1 ± 0.32	47.0 ± 3.34	2.3 ± 0.05	38.6 ± 0.26
23	10.1 ± 0.42	47.5 ± 3.33	2.4 ± 0.04	40.4 ± 0.32
24	10.1 ± 0.43	47.0 ± 2.83	2.2 ± 0.04	41.8 ± 0.16
25	9.0 ± 0.37	54.3 ± 2.59	2.4 ± 0.05	41.0 ± 0.00
26	8.2 ± 0.18	52.8 ± 3.20	2.5 ± 0.07	40.8 ± 0.31
27	9.5 ± 0.29	67.3 ± 2.78	2.7 ± 0.12	41.0 ± 0.00
28	8.8 ± 0.27	47.8 ± 1.30	2.4 ± 0.12	39.1 ± 0.13
29	11.3 ± 0.50	52.8 ± 1.44	2.3 ± 0.04	41.9 ± 0.13
30	8.5 ± 0.17	48.3 ± 1.70	2.5 ± 0.03	38.5 ± 0.19
31	10.7 ± 0.43	55.3 ± 2.95	2.6 ± 0.08	39.6 ± 0.46
32	9.2 ± 0.46	53.8 ± 1.79	2.3 ± 0.07	40.8 ± 0.75
33	10.0 ± 0.23	54.0 ± 2.68	2.7 ± 0.11	40.8 ± 0.75
34	9.8 ± 0.33	50.5 ± 1.76	2.4 ± 0.08	38.0 ± 0.00
35	11.4 ± 0.89	57.0 ± 2.42	2.5 ± 0.09	39.6 ± 0.32
36	9.3 ± 0.64	45.3 ± 2.33	2.4 ± 0.05	42.8 ± 0.45
37	9.3 ± 0.32	51.8 ± 3.75	2.3 ± 0.07	39.0 ± 0.42
38	10.6 ± 0.28	58.0 ± 2.12	2.5 ± 0.08	38.3 ± 0.16
39	9.1 ± 0.27	41.3 ± 1.84	2.5 ± 0.08	41.0 ± 0.00
40	10.7 ± 0.42	50.8 ± 4.03	2.6 ± 0.05	41.1 ± 0.13
41	10.6 ± 0.09	51.0 ± 2.08	2.6 ± 0.07	38.8 ± 0.31
42	10.9 ± 0.55	47.8 ± 1.65	2.6 ± 0.07	41.0 ± 0.00
Moneymaker	10.7 ± 0.38	68.8 ± 3.09	2.2 ± 0.08	41.7 ± 0.18

The morphogenetic response of different explants varied on the same combination of plant growth substances (Figures 3.1-3.4). Generally the percentage of explants which regenerated shoots was higher on stem internode and hypocotyl of cv. Moneymaker in comparison with leaf and cotyledon explants cultured on concentrations of BAP ≥ 4.4 μM after 4 weeks (Figures 3.1-3.4). Gunay and Rao (1980) reported that hypocotyl explants of a tomato hybrid were more responsive than cotyledon explants for producing shoots on a greater number of hormone combinations. Ohki et al. (1978) reported that the efficiency of shoot regeneration of two tomato cultivars was found to be dependent on the tissue used as the explant source, due to variation in types and concentrations of endogenous plant growth substances (BAP, IPA, and kinetin when combined with IAA). They found that the position within the organ from which the explant was excised also affected the efficiency of shoot regeneration and this was attributed to the uneven distribution of endogenous growth substances and inequality of histological constitution. The effects of organ maturity, types and concentrations of growth substances on the morphogenesis of explants were also reported (Ohki et al., 1978). The effects of explant source and donor plant conditions on shoot regeneration of tomato were examined by Frankenberger et al. (1981a). They found a significantly higher shoot-forming capacity in leaves that were closest to the apical meristem. In addition, the location of leaf explant tissue within the leaflet was reported to have a significant effect on shoot initiation on the plants (6-week-old) grown in the autumn season, but the leaf maturity had no effect on spring-grown plants (Frankenberger et al., 1981a). Cotyledon and leaf explants of tomato cultivars used in this study needed longer than 4 weeks to regenerate shoots on low BAP concentrations, however, the number of shoots regenerated on the higher concentrations was not different on all types of explants. In contrast, Gunay and Rao (1980) found that cotyledon explants produced

a greater number of shoots than hypocotyl explants. All of the above results emphasize a complex interaction of factors on the overall morphogenetic response.

Callus was induced on almost all the combinations of plant growth substances irrespective of the tomato genotype or auxin. Moneymaker explants rooted on NAA and most NAA and BAP combinations with variable percentages (Figures 3.1-3.4). However, 2,4-D in combination with BAP at similar concentration did not induce root regeneration in any type of explant of Moneymaker. These results agree with Kartha et al. (1976) who found a difference in the morphogenetic response as a result of changing the auxin used in combination with BAP on the leaf explants of tomato cv. Starfire. In addition, they reported that at concentration of 1.0-10 μM, IAA alone could induce a high frequency of shoot regeneration, whereas NAA alone at the same concentrations induced only a low frequency of regeneration. A combination of high concentrations of BAP and low concentrations of either NAA or 2,4-D induced shoot regeneration on all types of cv. Rootstock explants, but not on explants of cv. Moneymaker. This indicates an effect of the genotype on the morphogenetic response together with the auxin in use. Novak and Maskova (1979) noted induction of shoot regeneration on apical meristems of Moneymaker on media containing IAA with either BAP or kinetin, but IAA was added before autoclaving. Consequently the main effect might have been due to BAP or kinetin, confirming the results found in this study. In agreement with this study, they concluded that the combinations of NAA and BAP were not suitable for the development of plants from meristem explants of Moneymaker. Van Den Bulk et al. (1990) regenerated plants from leaf, cotyledon and hypocotyl explants of Moneymaker on MS supplemented with 4.5 μM zeatin and 0.5 μM IAA without comparing this combination with others. The combination of BAP and IAA was found to be optimum for the induction of shoot regeneration on all explant types of all tomato genotypes

tested by Carcia-Reina and Luque (1988) and Kartha et al. (1977).

The optimal combinations of plant growth substances for root regeneration varied considerably amongst the genotypes of tomato (Behki and Lesely, 1980; Frankenberger et al., 1981; Ohki et al., 1978; Carcia-Reina and Luque 1988; Kurtz and Lineberger 1983; Ziv et al., 1984; Tal et al., 1977; Paola et al., 1983). The morphogenetic response of the tomato cultivars used in this study was also affected by their genotypes. Ohki et al. (1978) noted a similar effect of genotype, concentration and type of plant growth substance on the morphogenesis of tomato.

Cytokinins were effective in inducing shoot regeneration from hypocotyl explants of tomato cv. Moneymaker, but the percentage of responding explants and the number of shoots produced per plant were higher on BAP in comparison with zeatin (Table 3.3). The superiority of BAP over zeatin on tomato regeneration was reported by Kartha et al. (1976). They found that multiple, vigourously growing shoots were regenerated by BAP on leaf explants of tomato cv. Stafire in 2 weeks, whereas only developing shoots were formed at a similar concentration of zeatin. Kinetin and BAP induced shoot regeneration on hypocotyl explants of cv. Rootstock cultured on B5 (Table 3.7) and the optimum concentration for shoot regeneration was different for the 2 cytokinins, and both induced regeneration of multiple shoots. The superiority of BAP over zeatin may be caused by different degradation rates of these substances in the medium or in the tissue. Blakesley and Lenton (1987) reported that shoot multiplication of *Gerbera jamesonii* was poor on zeatin compared to BAP. The investigation of the metabolism of these cytokinins in *Gerbera* callus revealed that the rate of zeatin oxidation was high and its level declined rapidly, in contrast to BAP, which persisted much longer in the callus.

Hypocotyl explants of Moneymaker were initiated onto 2,4-D media in an attempt to induce organogenic callus. Although organogenic tomato callus has been

reported (Padmanabhan, 1974; Behki and Lesely, 1976; Kartha et al., 1976; Tal et al., 1977; Ohki et al., 1978; Cassells, 1979), the regeneration of shoots was in a one-stage culture. Herman and Haas (1978) leaf-derived callus capable of shoot and leaf regeneration contained shoot or leaf fragments and no shoot organogenesis occurred in subcultured callus free of visible fragments of shoot and roots. Behki and Lesely (1980) reported that shoot regeneration from leaf callus of tomato was dependent on the composition of both induction and differentiation media as well as the duration of the induction period. They also found that the organogenesis of callus induced by 2,4-D was difficult when tested with a number of morphogenesis promoting media. The difficulty of regenerating shoots from Moneymaker callus might have been due to the use of 2,4-D to induce it. However, callus which was induced on combinations of 2,4-D with BAP produced shoots consistently (Behki and Lesely, 1980; Toyoda et al., 1989; and Garcia-Reina and Luque, 1988). De Langhe and De Bruijine (1976) regenerated normal shoots of tomato from callus 2 years after its establishment, when plants from which the cultures were derived were pretreated with chlormequat. Callus of *Lycopersicon peruvianum* also maintains its regenerative capacity for a long time (Thomas and Pratt, 1982). This regenerative capacity has been transferred to popular tomato cultivars by backcrossing (Koornneef et al., 1986; Koornneef et al., 1987). The genotype of tomato was also reported to affect the organogenesis of callus induced from its explants (Meredith, 1979). The regeneration of shoots from Moneymaker callus, and subsequently from cell suspension cultures would help in the selection of tomato plants resistant to *Xanthomonas campestris* pv. *vesicatoria* from cell suspension cultures of this cultivar.

Histological studies on hypocotyl explants of Moneymaker revealed that cell division occurred on the cut surface and that these developed into globular calli with nodular meristematic cell areas. There was a disorganized vascular system distributed

around the globular surface. These meristematic areas were shoot primordia which were established 16 days after culture initiation. These results are in agreement with Cassells (1979), Garcia-Reina and Luque (1988) and De Langhe and De Bruijne (1976). In contrast, Compton and Veilleux (1991) reported direct shoot regeneration from pedicel explants of tomato on which a very small amount of callus was formed. Most of the somaclones regenerated from Moneymaker in this study were diploid, fertile and could be used for tomato improvement if useful variation had been induced on these somaclones. Organogenetic tomato callus however has resulted in a high frequency of abnormalities (Vnuchkova, 1977; reviewed by Kut et al., 1984; Koorneef et al., 1989; O'Connell et al., 1986; Meredith, 1979).

The results presented in Section 3.1.10 added further strength to the proposed use of somaclonal variation to recover new genetic variability from existing tomato varieties (Buiatti et al., 1985; Evans et al., 1984; Evans and Sharp, 1983; Van Den Bulk, 1990; Koornneef et al., 1989; O'Connell et al., 1986; Evans, 1989; Barden et al., 1986). The potential of somaclonal variation in tomato improvement was demonstrated by Evans (1989). He developed two cultivars (one with high solids and the other resistant to *Fusarium* race 2) from somaclones regenerated from leaf explants of cv. UC82B. Changes in the morphology and genetic make up observed on the somaclones of both cultivars used was also reported by others (Evans and Sharp, 1983; Koornneef et al., 1989; O'connell et al., 1986; Van Den Bulk, 1990). Some of these workers reported mutations in single genes, but most frequently tetraploid somaclones were obtained. The ploidy levels of tomato somaclones depended on sources of explants. O'Connell et al. (1986) and Koorneef et al. (1989) found that the somaclones which were regenerated from leaf explants were predominantly diploid and the regenerants from established callus cultures or protoplasts were tetraploid. The explant type was found to influence the frequency of polyploidy

of somaclones regenerated from Moneymaker (Van Den Bulk, 1990). Endoreduplication was one of the mechanisms responsible for polyploidy in potato (Sree Ramulu, 1986). The tetraploid somaclones of Moneymaker did not produce seeds as a result polyploidy was not investigated in the progenies of these tetraploid somaclones. Sterility of tetraploid tomato somaclones was reported by Evans and Sharp (1983). In contrast, Van Den Bulk (1990) reported that tetraploidy of somaclones regenerated from Moneymaker was transmitted to their progenies, but not the phenotypic changes which might have resulted partially from non-heritable epigenetic mutations. Several examples of recessive and dominant monogenic mutations obtained through tissue culture of tomato resulting in various phenotypic changes and disease resistance have been reported (Evans and Sharp, 1983; Evans, 1989; Van Den Bulk, 1990; Shahin and Spivey, 1986; Barden et al., 1986). The regeneration system which was used on both tomato cultivars in this study proved to be mutagenic and induced genetic as well as morphogenetic changes. The somaclones from Moneymaker and Rootstock were assessed against two pathogens, powdery mildew and *Xanthomonas campestris* pv. *vesicatoria* together with their parental cultivars to find out whether changes in the state of their resistance had occurred. Results of this screening programme will be discussed in the following chapters.

Chapter (4)

Tomato Leaf Spot Disease Caused by *Xanthomanas campestris* pv. *vesicatoria* (*Xcv*)

4.1 Results

The *Xcv* strains used in this study were the wild type (E3), the non-pathogenic mutants (E1004 and E1141) and the pathogenic mutant (E1141(p6AD4)) described in Section 2.5.1. The growth of the pathogenic and non-pathogenic *Xcv* strains *in vitro* and *in planta* were assessed. Host-pathogen interaction was studied in resistant and susceptible genotypes of tomato and pepper. Comparative population trends, cell leakage and symptom development were assessed for E3, E1141 and E1141(p6AD4) strains of *Xcv* using two inoculum concentrations of each. The killing abilities of these strains on cells of tomato (cv. Rootstock) from different growth stages of suspension culture were investigated *in vitro*. Low molecular toxin production in the co-culture medium between E3 and the tomato cells was also investigated. Somaclones of tomato cvs Rootstock and Moneymaker were tested for resistance to the *Xcv* strain E3.

4.1.1 Materials and Methods

The preparation of *Xcv* inocula from overnight cultures and the inoculation and incubation conditions of plants (tomato or pepper), unless otherwise described, were as described in Section 2.5.3 and 2.5.4 respectively. All tomato seedlings used in this study were 4-weeks old and the pepper seedlings were 6 weeks-old. Assessment of cell leakage of infiltrated leaves and living bacteria *in planta* and *in vitro* were done as described in Sections 2.5.5 and 2.5.6 respectively. Leaf spot symptoms in resistant and susceptible plants of tomato and pepper were

assessed using 1-6 and 0-5 scales respectively where 0 represents no disease symptoms and 5 represents severe leaf spot symptoms in the susceptible plants and 6 represents a typical hypersensitive reaction in the resistant ones.

4.1.2 Growth of Virulent and Avirulent *Xcv* Strains *in vitro*

E1141 and E1004 are non-pathogenic mutants of *Xcv* strain E3 (wild type). The three strains were grown *in vitro* to see if any variation in growth was present between them and to test the effect of two media (i.e. NYGB which is called the rich medium, Appendix 1; and Modified Watanabe broth which is called the minimum medium, Appendix 2) on the growth of these strains. Three replicates of 100 ml of each medium were inoculated with *Xcv* by adding 1 ml of 1.1 X 10^7 cfu ml^{-1}. The growth of the three strains E3, E1004 and E1141 was similar in NYGB (Figure 4.1) as well as in modified Watanabe broth (Figure 4.2), but the growth rate was higher in NYGB and the doubling time during the exponential phase was two hours for all strains in NYGB. However, in modified Watanabe broth the growth rate of the three strains was slower reaching half of that in NYGB after 24 hours and the doubling time was 4 hours (Figures 4.1 and 4.2). E3 when cultured in NYGB multiplied rapidly up to 2.22 X 10^9 cfu ml^{-1} 18 h after the culture and this population density was equivalent to 2.6 units at 600 nm but when E3 was cultured in modified Watanabe broth for the same period it multiplied up to 1.31 X 10^9 cfu ml^{-1} and this equivalent to 1-1.18 units at 600 nm (Appendix 3 and 4).

4.1.3 Evaluation of Different *Xcv* Inoculation Methods on Tomato and Pepper Seedlings

The Inoculation method frequently has an important effect on the development of disease symptoms. A consistent inoculation method was necessary for host-parasite interaction studies on different host genotypes, and for the screening

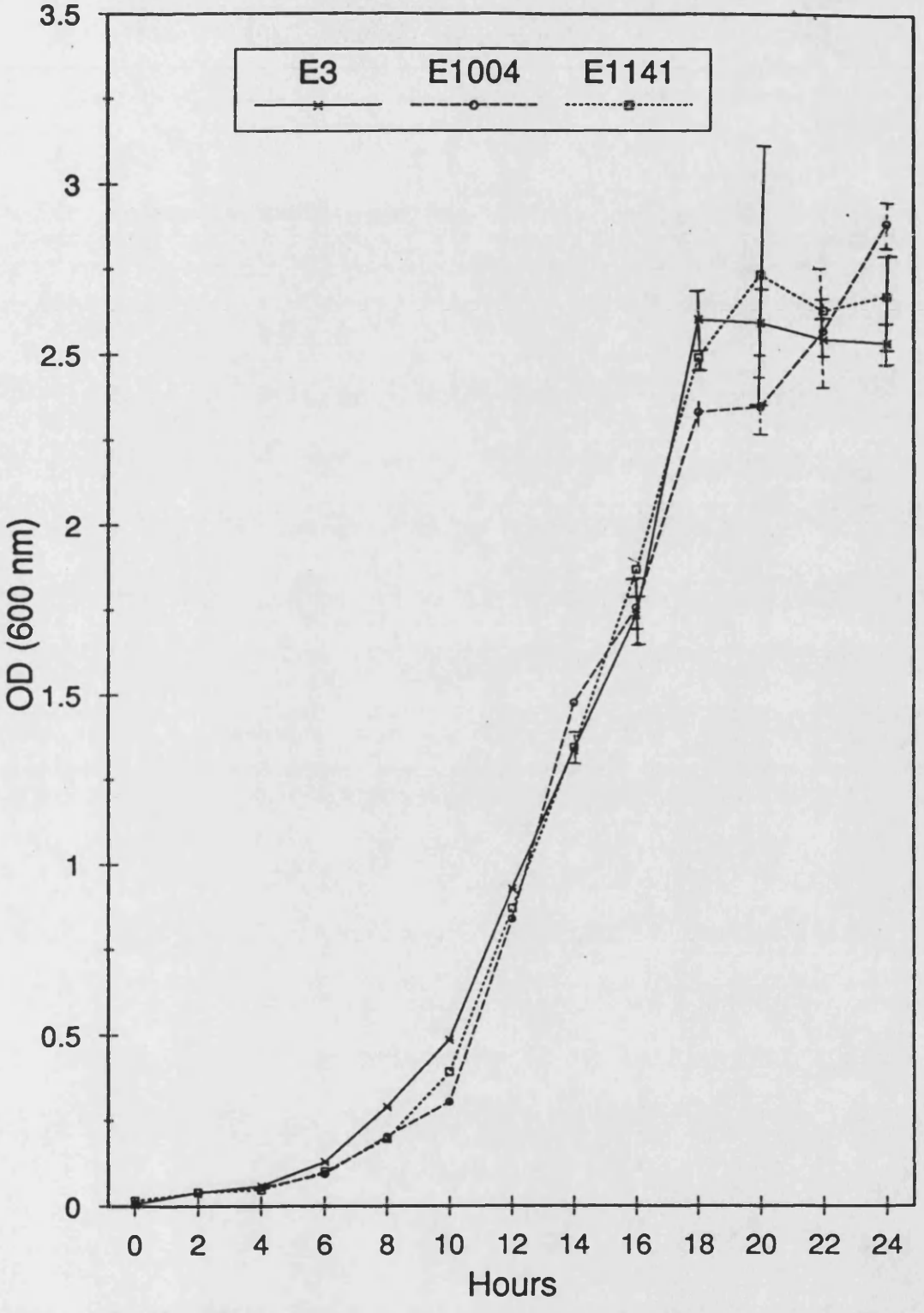


Figure 4.1: Growth of *Xanthomonas campestris* pv *vesicatoria* strains E3, E1004 and E1141 in nutrient yeast glycerol broth (NYGB)

100 ml of NYGB was inoculated with 1.1 X 10^7 cfu ml^{-1} of E3, E1004 and E1141 and incubated at 30 °C on an orbital shaker. Means of 3 replicates are shown with the stadard error bars.

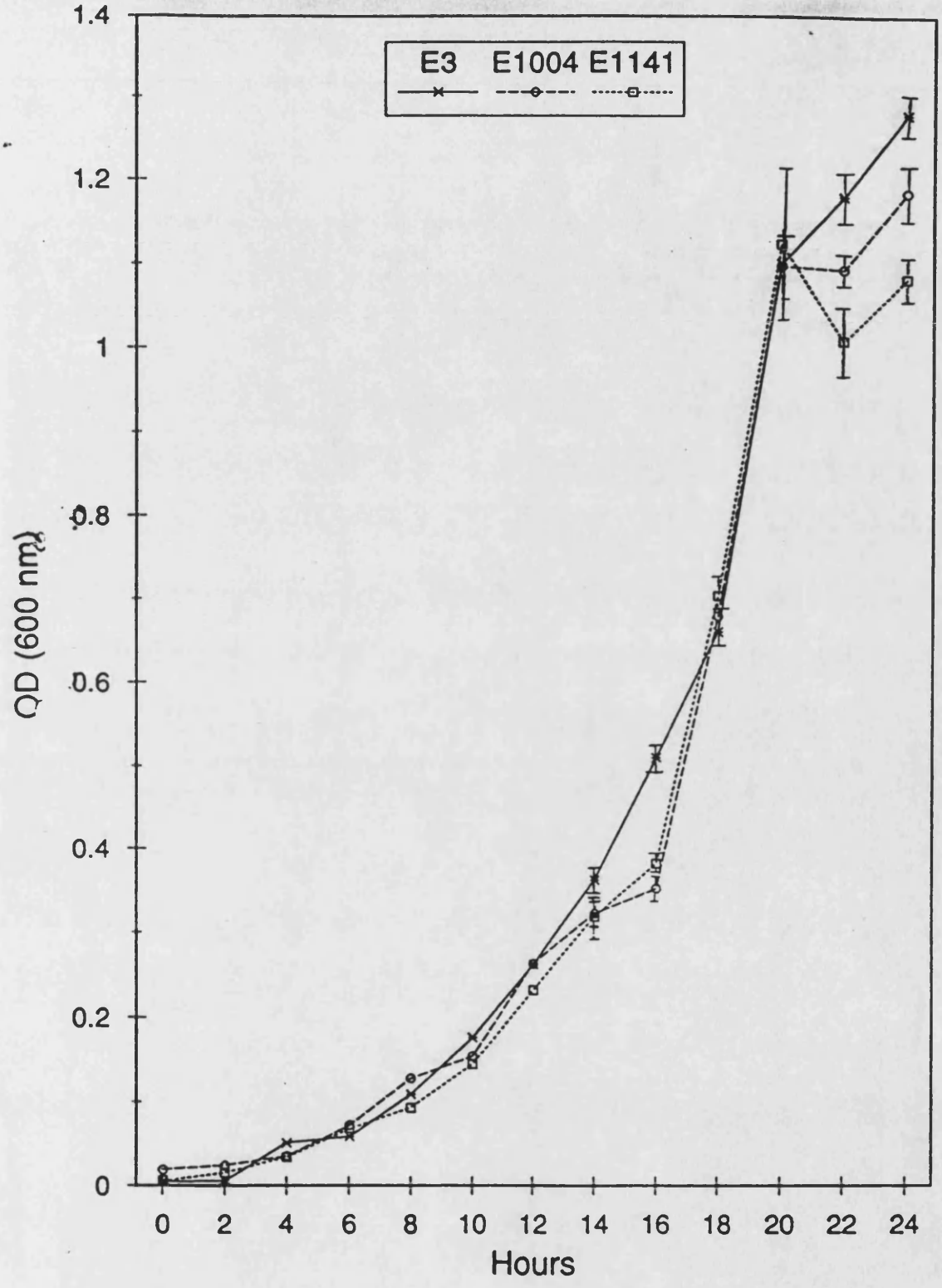


Figure 4.2: Growth of *Xanthomonas campestris* pv *vesicatoria* strains E3, E1004 and E1141 in modified watanabe broth

100 ml of NYGB was inoculated with 1.1 X 10^7 cfu ml^{-1} of E3, E1004 and E1141 and incubated at 30 °C on an orbital shaker. Means of 3 replicates are shown with the stadard error bars.

of tomato somaclones against the pathogen. Four replicate seedlings of susceptible tomato (cv. Moneymaker) and pepper (cv. ECW) were inoculated with a suspension of E3 (10^8 cfu ml^{-1}) by three methods i.e. spraying, hand rubbing plus spraying and leaf infiltration and the disease symptoms were assessed one and two weeks after inoculation.

Leaf infiltration was the only method which showed water soaking symptoms 2 days after inoculation. The colour of the infiltrated area changed to brown at the middle after 4 days and started to dry up on both tomato and pepper seedlings (Plates 4.1 and 4.2, Tables 4.1 and 4.2). Tomato plants inoculated by hand rubbing plus spraying of leaves developed symptoms after 2 weeks, presumably due to the damage to the hairy leaf surface (Table 4.1). However, pepper seedlings which were inoculated by the same method did not develop disease symptoms during the same period, but the leaves of some plants showed curling on the inoculated areas (Table 4.2). Hand rubbing is highly variable between plants (Table 4.2) and abrasives (suspension celite (1% w/v) and carborundum) were rubbed on the surface of tomato leaves to give uniform damage, but there was no difference between inoculated leaves and the control treatment in which the colour turned brown and dried after 10 days. Spraying alone as a method of inoculation was not suitable on tomato or pepper.

4.1.4 Comparison of Disease Symptoms on Susceptible and Resistant Pepper Genotypes Using Pathogenic and Non-pathogenic Strains of *Xcv*

Disease symptoms were induced in a susceptible pepper (ECW) 4 days after infiltration with *Xcv* strain E3 at 10^8 cfu ml^{-1}. The interaction between resistant and susceptible pepper genotypes and pathogenic *Xcv* strains (E3 and E1141) and non-pathogenic *Xcv* strain E1141 was investigated and disease symptoms were compared in both genotypes. Four seedlings of pepper cv. ECW (susceptible) and

Plate 4.1: Leaf spot symptoms on pepper cv. Early Calwonder leaves 4 day after leave infiltration with *Xcv* strain E3

Plate 4.2: Leaf spot symptoms on tomato cv. Moneymaker leaves 4 day after infiltration with *Xcv* strain E3


4.1


4.2

Table 4.1: Evaluation of different inoculation methods of *Xcv* strain (E3) on leaves of tomato (cv. Moneymaker)

Inoculation Methods	Time (weeks)	Score
Spray by D. water	1	0.00
Spray by *Xcv*		0.00
Hand rubbing + Spray(*Xcv*)		0.00
Leaf infiltration(*Xcv*)		3.33 ± 0.25
Spray by D. water	2	0.00
Spray by *Xcv*		0.00
Hand rubbing + Spray(*Xcv*)		3.02 ± 0.26
Leaf infiltration(*Xcv*)		3.90 ± 0.11

Seedlings of tomato cv. Moneymaker were inoculated with E3 (10^8 cfu ml^{-1}) and incubated at 25 ± 1 ^{0}C, 88-92% rh, 16 h photoperiod and light intensity of 180-250 μE m^{-2} s^{-1}. Plants were assessed for the development of disease symptoms after one and two weeks of inoculation using the following scale:

0 = No symptoms; 1 = Lesions green, water-soaked, translucent, or just begining to turn brown, but with no chlorosis; 2 = Lesions brown and with small chlorotic halos; 3 = Lesions brown and conspicuous chlorosis around; 4 = Extensive chlorosis, affected portions may have turned brown and dried out, pronounced epinasty of leaflets and/or leaves.

Data are the means and standard errors of 4 replicates for each treatment.

Table 4.2: Evaluation of different inoculation methods of *Xcv* strain (E3) on leaves of pepper (cv. ECW)

Inoculation Methods	Time (weeks)	Score
Spray by D. water	1	0.00
Spray by *Xcv*		0.00
Hand rubbing + Spray(*Xcv*)		0.00
Leaf infiltration(*Xcv*)		4.66 ± 0.08
Spray by D. water	2	0.00
Spray by *Xcv*		0.00
Hand rubbing + Spray(*Xcv*)		0.00
Leaf infiltration(*Xcv*)		5.00

Seedlings of pepper cv. ECW were inoculated with E3 (10^8 cfu ml^{-1}) and incubated at 25 ± 1 ^{0}C, 88-92% rh, 16 h photoperiod and light intensity of 180-250 μE m^{-2} s^{-1}. Plants were assessed for the development of disease symptoms after one and two weeks of inoculation using the following scale:

0 = No symptoms; 1 = Lesions green, water-soaked, translucent, or just begining to turn brown, but with no chlorosis; 2 = Lesions brown and with small chlorotic halos; 3 = Lesions brown and conspicuous chlorosis around; 4 = Extensive chlorosis, affected portions may have turned brown and dried out, pronounced epinasty of leaflets and/or leaves; 5 = Leaf drop.

Data are the means and standard errors of 4 replicates for each treatment.

ECW-10R (resistant, carrying Bs1 resistance gene) were infiltrated with a suspension (10^8 cfu ml^{-1}) of each of the three strains of *Xcv* (i.e. E3, E1141 and E1141(p6AD4). ECW plants infiltrated with water or any of the bacterial strains showed no reaction 24 h after infiltration whereas ECW-10R plants infiltrated with E3 and E1141(p6AD4) showed a hypersensitive response (HR) (Plate 4.3). Infiltrated areas collapsed and the leaves fell after 36 h. There were no symptoms induced by the non-pathogenic strain E1141 on ECW-10R in the same period (Plate 4.3).

4.1.5 Comparative Population Growth, Cell Leakage and Disease Symptoms on the Leaves of Pepper ECW Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

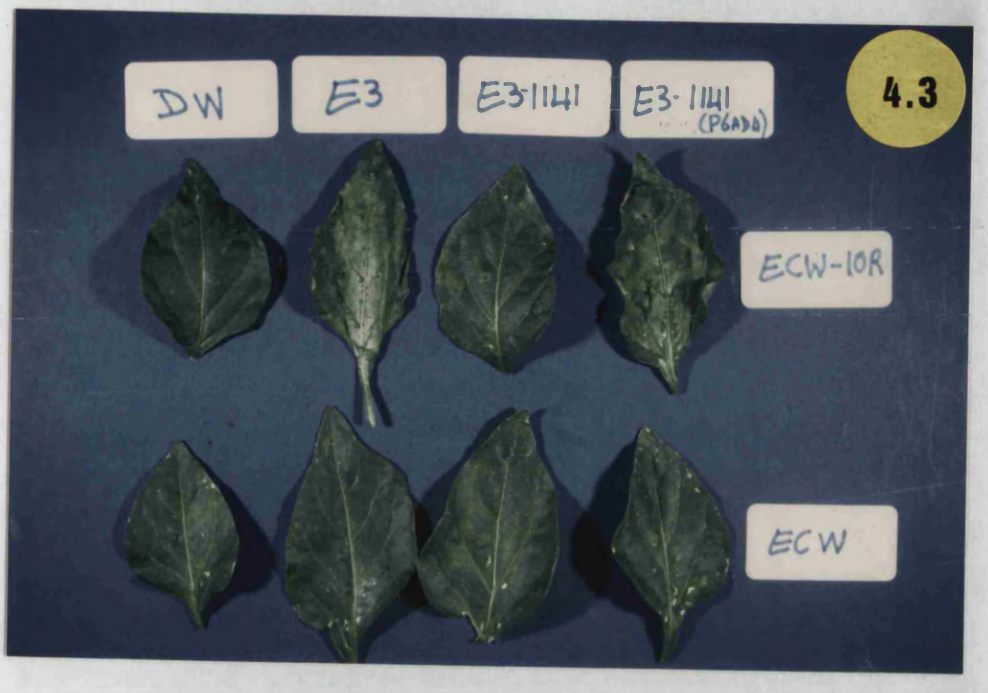
Seedlings of pepper cv. ECW were infiltrated with either 10^6 or 10^8 cfu ml^{-1} of *Xcv* strains E3, E1141, and E1141(p6AD4). The population growth of the two inoculum concentrations of these pathogenic and non-pathogenic *Xcv* strains on the leaves of five replicate seedlings of cv. ECW per concentration were investigated. Cell leakage and symptom development were also assessed.

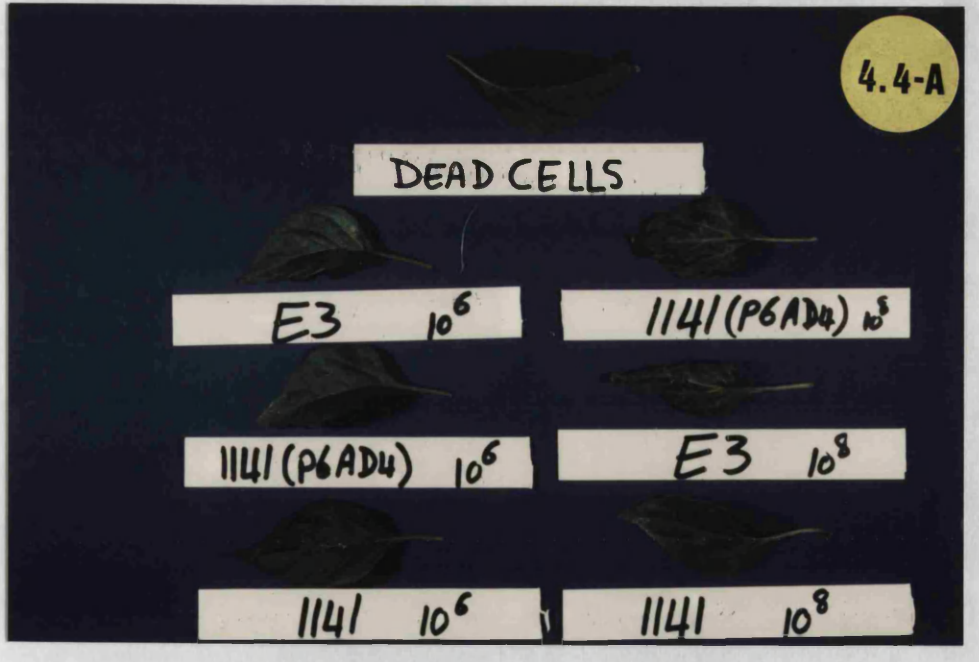
4.1.5.1 Symptoms of Leaf Spot Disease on Pepper cv. ECW Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

Symptoms caused by *Xcv* strains E3, E1141(p6AD4) and E1141 on the leaves of pepper cv. ECW infiltrated with two inoculum concentrations of each strain were scored according to 0-5 scale where 0 represented no symptoms and 5 represented leaf drop (severe disease symptoms). Infiltrated areas showed water-soaking symptoms which disappeared after 4-6 h in all the treatments, including the control. Pepper leaves which were infiltrated with high inoculum concentrations (10^8 cfu ml^{-1}) of E3 and E1141(p6AD4) showed water-soaking symptoms after 2 days, became transparent and started to dry at the middle and

Plate 4.3: Leaf spot symptoms on susceptible pepper (cv. Early Calwonder) and resistant pepper (cv. ECW-10R) genotypes 24 after hours of infiltration with different Xcv strains

Plate 4.4a: Symptoms of leaf spot disease on pepper (ECW) leaves 4 days after infiltration with 10^6 and 10^8 cfu ml^{-1} of E3 , E1141 and E1141(p6AD4)


DW
E3
E3-1141
E3-1141 (P6AD4)
4.3
ECW-10R
ECW


4.4-A
DEAD CELLS
E3 10^6
1141(P6AD4) 10^8
1141(P6AD4) 10^6
E3 10^8
1141 10^6
1141 10^8

turned brown after 3 to 4 days (Table 4.3, Plate 4.4a). However, low inoculum concentrations of E3 and E1141(p6AD4) took a longer time to show symptoms and developed water-soaking symptoms 6 days and browning 8 days after infiltration (Plates 4.4b-d). No symptoms developed on the leaves of pepper cv. ECW infiltrated by the low and high inoculum concentrations of the non-pathogenic *Xcv* strain E1141 (Table 4.3).

The symptoms were analysed according to a split plot design after square root transformation. Disease symptoms varied significantly ($p < 0.001$) between the different inoculum concentrations of *Xcv* strains. The length of time after infiltration had also affected symptoms development significantly ($p < 0.001$) (Appendix 5).

4.1.5.2 Conductivity of Pepper cv ECW Leaves Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

Conductivities of pepper ECW tissues infiltrated with different strains of *Xcv* (E3, E1141, and E1141(p6Ad4)) using two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) are presented in Figure 4.3. The conductivity of leaves increased rapidly when infiltrated with high inoculum concentrations (10^8 cfu ml^{-1}) of the pathogenic strains E3 and E1141(p6AD4) (Figure 4.3). However, E1141(p6AD4) was slower than E3 and the conductivity of the latter was 76.96% 2 days after infiltration compared to 78.38% of E1141(p6AD4) 3 days after infiltration (Figure 4.3). The conductivity of leaves infiltrated with E3 and E1141(p6AD4) at 10^6 cfu ml^{-1} increased steadily with time from 20.5 to 88.34% and 21.1 to 85.74% respectively in 11 days (Figure 4.3). Cell leakage of plant tissues infiltrated with the non-pathogenic strain E1141 was low irrespective of the inoculum concentration used although it was slightly higher than that of the control. Generally, the conductivity of pepper tissue infiltrated by E3 and E1141(p6AD4) followed

Plate 4.6a: Symptoms of leaf spot disease on tomato (cv. Hawaii 7998) leaves 3 days after infiltration with 10^6 and 10^8 cfu ml^{-1} of E3, E1141 and E1141(p6AD4)

Plate 4.6b: Symptoms of leaf spot disease on tomato (cv. Hawaii 7998) leaves 6 days after infiltration with 10^6 and 10^8 cfu ml^{-1} of E3, E1141 and E1141(p6AD4)


DEAD CELLS
E3 10^6
E3 10^8
8 DAYS
4.4-B


DEAD CELLS
1141 (P6AD4) 10^6
1141 (P6AD4) 10^8
8 DAYS
4.4-C


DEAD CELLS
1141 10^6
1141 10^8
8 DAYS
4.4-D

Table 4.3: Leaf spot symptoms on susceptible pepper (cv. ECW) infiltrated with *Xcv* strains E3, E1141 and E1141(p6AD4)

Days	Treatments						
	Dead E3 (10^8)	E3 (10^6)	E3 (10^8)	E1141 (p6AD4) (10^6)	E1141 (p6AD4) (10^8)	E1141 (10^6)	E1141 (10^8)
0	0	0	0	0	0	0	0
1	0	0	1.3 ± 0.18	0	1.0	0	0
2	0	0	1.8 ± 0.18	0	1.8 ± 0.20	0	0
3	0	0	3.4 ± 0.22	0	3.0 ± 0.29	0	0
4	0	0	3.8 ± 0.18	0	3.2 ± 0.20	0	0
6	0	1.2 ± 0.18	5.0	0.8 ± 0.18	5.0	0	0
8	0	3.4 ± 0.22	5.0	3.2 ± 0.18	5.0	0	0
11	0	3.8 ± 0.20	5.0	3.4 ± 0.22	5.0	0	0

Seedlings of pepper cv. ECW were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3, E1141 and E1141(p6AD4) and icubated at 25 ± 1 ^{0}C, 88-92% rh, 16 h photoperiod and light intensity of 180-250 $\mu E\ m^{-2}\ s^{-1}$. Plants were assessed for the development of disease symptoms almost every day using the following scale:

0 = No symptoms; 1 = Lesions green, water-soaked, translucent, or just begining to turn brown, but with no chlorosis; 2 = Lesions brown and with small chlorotic halos; 3 = Lesions brown and conspicuous chlorosis around; 4 = Extensive chlorosis, affected portions may have turned brown and dried out, pronounced epinasty of leaflets and/or leaves; 5 = Leaf drop.

Data are the means and standard errors of 5 replicates for each treatment.

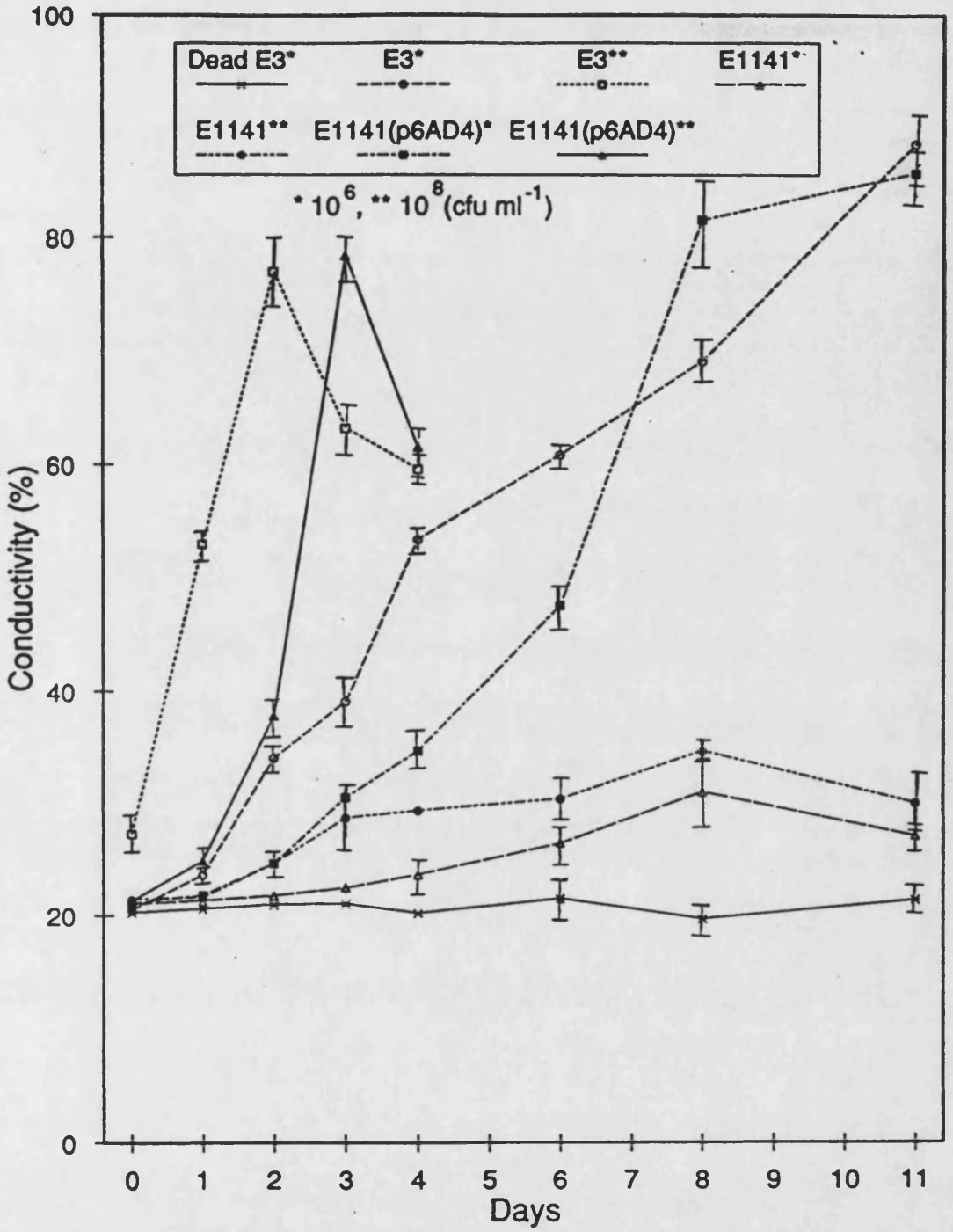


Figure 4.3: Conductivity of pepper (cv. ECW) leaves infiltrated with *X. campestris* pv. *vesicatoria* strians E3, E1141 and E114(p6AD4)

Leaves of pepper cv. ECW seedlings were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3 (wild type), E1141 (nonpathogenic mutant) and E1141(p6AD4) (pathogenic mutant) and incubated in a growth chamber at 25 °C, 89-92% rh, 16 h day and 180-250 µE m^{-2} s^{-1} light. Fifteen leaf discs (4mm diamter) were taken per replicate. Means of 5 replicates are shown with the standard error bars.

the same pattern of bacterial population growth. High cell leakage was caused when the population of the pathogenic bacteria was at its highest level (Figure 4.3 and Figure 4.4).

4.1.5.3 Population Growth of *Xcv* Strains E3, E1141 and E1141(p6AD4) in the Leaves of Susceptible Pepper ECW

The populations of all *Xcv* strains increased in the pepper (ECW) tissues with time irrespective of their pathogenicity. The rate of multiplication was higher when low inoculum concentrations (10^6 cfu ml^{-1}) of E3 and E1141(p6AD4) were infiltrated into the leaves of pepper seedlings (Figure 4.4). The population of these *Xcv* strains increased 400 fold 2 days after infiltration with low inoculum concentrations (10^6 cfu ml^{-1}). However, population growth of E1141 infiltrated at a low concentration increased approximately 100 fold in 3 days and remained fairly stable until the termination of the experiment (Figure 4.4). When high inoculum concentrations (10^8 cfu ml^{-1}) of E3 and E1141(p6AD4) were infiltrated in pepper (ECW) leaves, the population of these pathogenic bacteria increased rapidly reaching 400 fold each compared to 200 fold for the non-pathogenic *Xcv* strain E1141 3 days after infiltration (Figure 4.4). E3 and E1141(p6AD4) populations of the high inoculum concentrations declined after 4 days because the infiltrated leaves collapsed and fell off. Although the growth rate was higher when E3 and E1141(p6AD4) were infiltrated in the leaves of cv. ECW using a low inoculum concentration (10^6 cfu ml^{-1}), the population took 8 days to grow to the same level as the high inoculum concentration (10^8 cfu ml^{-1}) 4 days after infiltration (Figure 4.4). There was little difference in the growth of both inocula of the non-pathogenic strain E1141 3 days after infiltration.

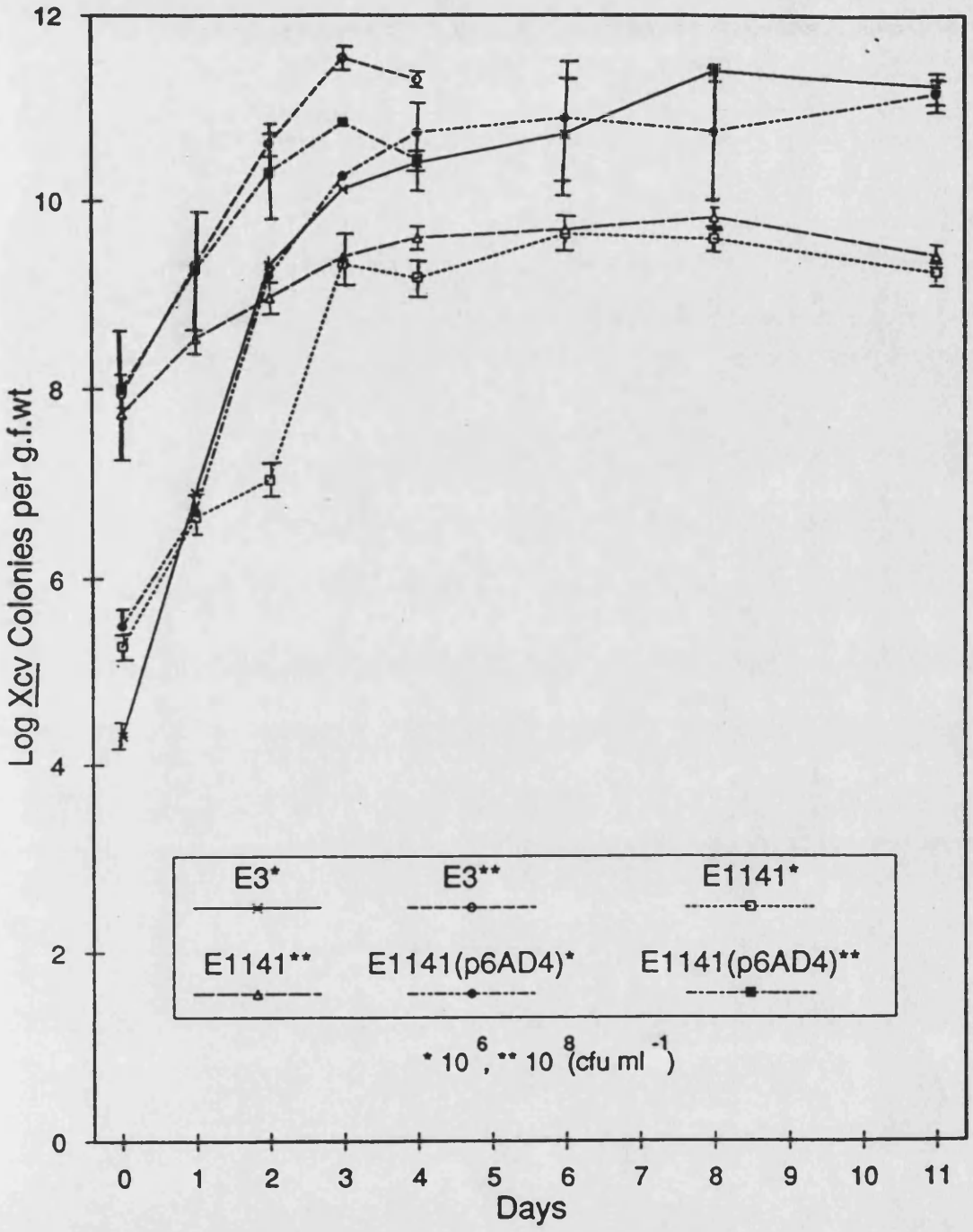


Figure 4.4: Comparative population trends of *X. campestris* pv. *vesicatoria* strains E3, E1141 and E114(p6AD4) infiltrated in leaves of pepper cv. ECW

Leaves of pepper cv. ECW seedlings were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3 (wild type), E1141 (nonpathogenic mutant) and E1141(p6AD4) (pathogenic mutant) and incubated in a growth chamber at 25 °C, 89-92% rh, 16 h day and 180-250 µE m^{-2} s^{-1} light. Fifteen leaf discs (4mm diamter) were taken per replicates. Means of 5 replicates are shown with the standard error bars.

4.1.6 Comparative Population Trends, Conductivity and Symptoms of Tomato cv. Rootstock Infiltrated with *Xcv* Strains E3, E1141, and E1141(p6AD4)

Seedlings of tomato cv. Rootstock were infiltrated with 2 inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3, E1141 and E1141(p6AD4). Five replicate seedlings were infiltrated with each inoculum concentration and the population growth, conductivity and symptom development were assessed. Angular transformation of conductivity readings and log transformation of bacterial numbers were done before the analysis of the data according to split plot design where plants represented the whole block and sampling times represented subplots.

4.1.6.1 Symptoms of Leaf Spot Disease on Tomato cv. Rootstock Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

Disease symptoms were scored following 0-4 scale where 0 represents no symptoms and 4 represents severe disease symptoms. The non-pathogenic E1141 strain did not cause any symptoms of leaf spot disease on the leaves of tomato cv. Rootstock infiltration with either inoculum concentration (10^6 and 10^8 cfu ml^{-1}) (Table 4.4). However, E3 (10^8 cfu ml^{-1}) showed water-soaking symptoms on tomato leaves 24 h after infiltrated and the tissue became necrotic 3 days after infiltration, but E1141(p6AD4) infiltrated at high inoculum concentration showed water soaking symptoms 2 days after infiltration and small necrotic areas developed 3 days after infiltration (Plate 4.5a). Disease symptoms were analysed according to split plot design after square root transformation. Symptoms caused by different inoculum concentrations of *Xcv* strains were significantly different ($p < 0.01$) (Appendix 6). Lower inoculum concentrations (10^6 cfu ml^{-1}) of E3 and E1141(p6AD4) showed water-soaking areas 4 days after infiltration and the

Table 4.4: Leaf spot symptoms on susceptible tomato (cv. Rootstock) infiltrated with *Xcv* strains E3, E1141 and E1141(p6AD4)

Days	Treatments						
	Dead E3 (10^8)	E3 (10^6)	E3 (10^8)	E1141 (p6AD4) (10^6)	E1141 (p6AD4) (10^8)	E1141 (10^6)	E1141 (10^8)
0	0	0	0	0	0	0	0
1	0	0	1.0	0	0	0	0
2	0	0	1.6 ± 0.22	0	0	0	0
3	0	0	3.5 ± 0.22	0	1.4 ± 0.18	0	0
4	0	1.0	3.8 ± 0.19	1.6 ± 0.24	3.2 ± 0.20	0	0
6	0	3.4 ± 0.24	3.8 ± 0.19	3.2 ± 0.18	3.6 ± 0.22	0	0

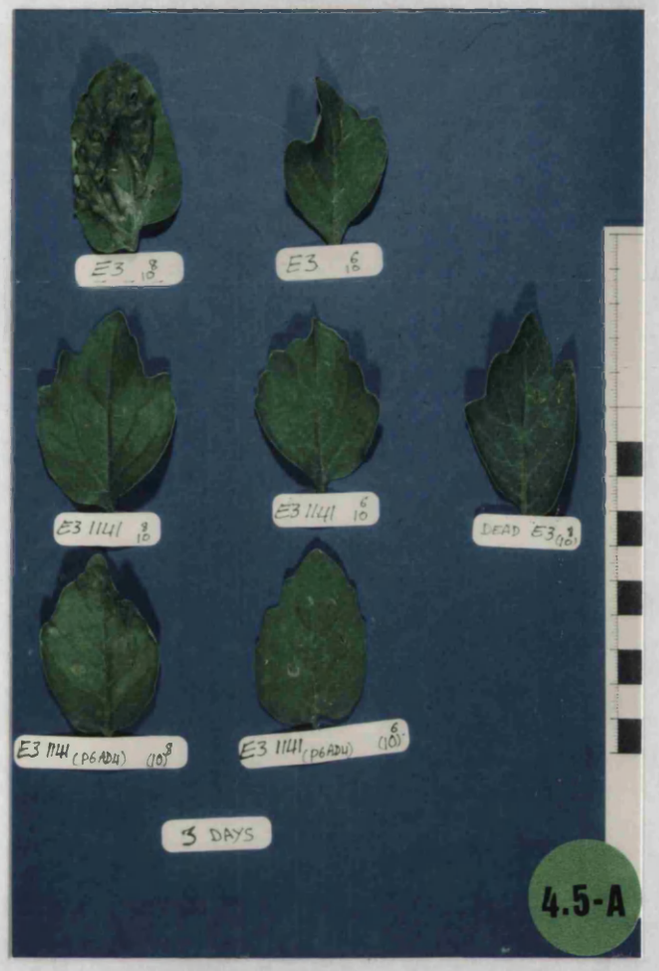
Seedlings of tomato cv. Rootstock were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3, E1141 and E1141(p6AD4) and icubated at 25 ± 1 ^{0}C, 88-92% rh, 16 h photoperiod and light intensity of 180-250 μE m^{-2} s^{-1}. Plants were assessed for the development of disease symptoms almost every day using the following scale:

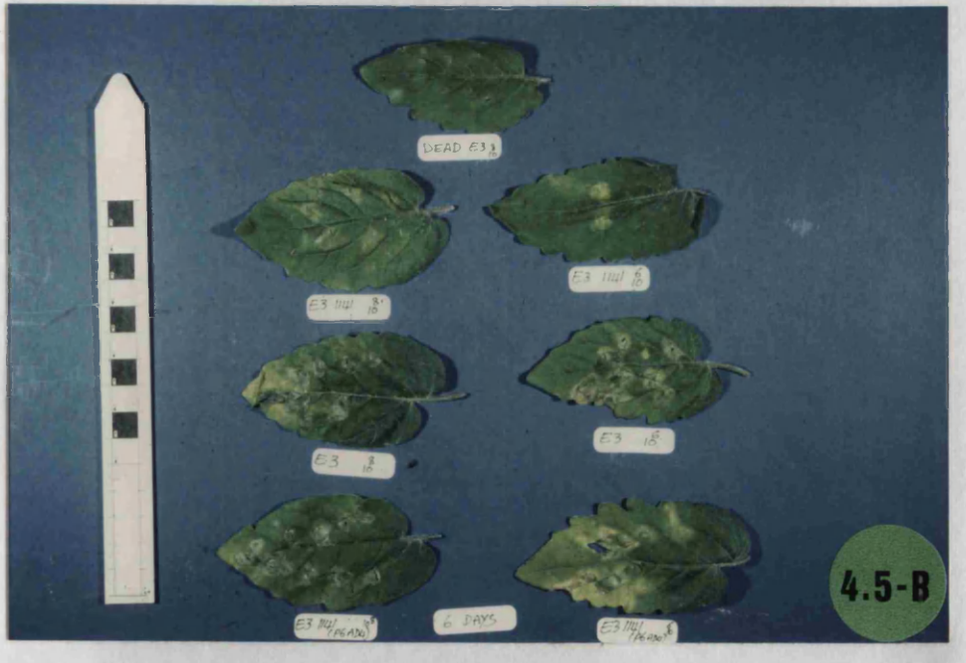
0 = No symptoms; 1 = Lesions green, water-soaked, translucent, or just begining to turn brown, but with no chlorosis; 2 = Lesions brown and with small chlorotic halos; 3 = Lesions brown and conspicuous chlorosis around; 4 = Extensive chlorosis, affected portions may have turned brown and dried out, pronounced epinasty of leaflets and/or leaves.

Data are the means and standard errors of 5 replicates for each treatment.

Plate 4.5a: Symptoms of leaf spot disease on tomato (cv. Rootstock) leaves 3 days after infiltration with 10^6 and 10^8 cfu ml^{-1} of E3, E1141 and E1141[p6AD4]

Plate 4.5b: Symptoms of leaf spot disease on tomato (cv. Rootstock) leaves 6 days after infiltration with 10^6 and 10^8 cfu ml^{-1} of E3, E1141 and E1141[p6AD4].


E3 8/10
E3 6/10
E3 11411 8/10
E3 11411 6/10
DEAD E3 1/10
E3 11411 (P6ADU) (10) 8
E3 11411 (P6ADU) (10) 6
3 DAYS
4.5-A


DEAD E3
E3 11411 8/10
E3 11411 6/10
E3 8/10
E3 18
E3 11411 (P6ADU)
6 DAYS
E3 11411 (P6ADU)
4.5-B

infiltrated tissues were necrotic 6 days after infiltration. The length of time after infiltration had a significant ($p < 0.5$) effect on symptoms development (Appendix 6, Plate 4.5b).

4.1.6.2 Conductivity of Leaves of Tomato cv. Rootstock Infiltrated with *Xcv* Strains E3, E1141, E1141(p6AD4)

When high inoculum concentrations of *Xcv* were infiltrated in leaves of cv. Rootstock, the increase in cell leakage caused by E3 was higher than that of E1141(p6AD4) and E1141 (Figure 4.5). Cell leakage in tomato cv. Rootstock leaves varied significantly with inoculum concentrations of *Xcv* strains ($p < 0.001$) (Appendix 7). Four days after infiltration, cell leakage of E3 and E1141(p6AD4) (10^8 cfu ml^{-1}) increased 70% and 57.3% respectively compared to 28.32% of E1141 (Figure 4.5). Cell leakage of tomato leaves infiltrated with 10^6 cfu ml^{-1} of the three strains of *Xcv* as well as that infiltrated with dead E3 cells increased significantly with time ($p < 0.001$) (Appendix 7), because the plants of this tomato cultivar showed physiological stress under incubation conditions. Cell leakage was higher in the tomato leaves infiltrated with 10^6 cfu ml^{-1} of E3 and E1141(p6AD4) compared to the non-pathogenic *Xcv* strain E1141 6 days after infiltration (Figure 4.5).

4.1.6.3 Population Growth of *Xcv* Strains E3, E1141 and E1141(p6AD4) in the Leaves of Susceptible Tomato cv. Rootstock

Population growth of *Xcv* strains E3, E1141 and E1141(p6AD4) in the susceptible tomato cv. Rootstock was similar to that in the susceptible pepper ECW. Population increase was significantly different with time, inoculum concentration as well as the interaction of time and the bacterial strains ($p < 0.001$, Appendix 8). The population was higher when high inoculum concentrations (10^8 cfu ml^{-1}) of the pathogenic strains E3 and E1141(p6AD4) were used, but the

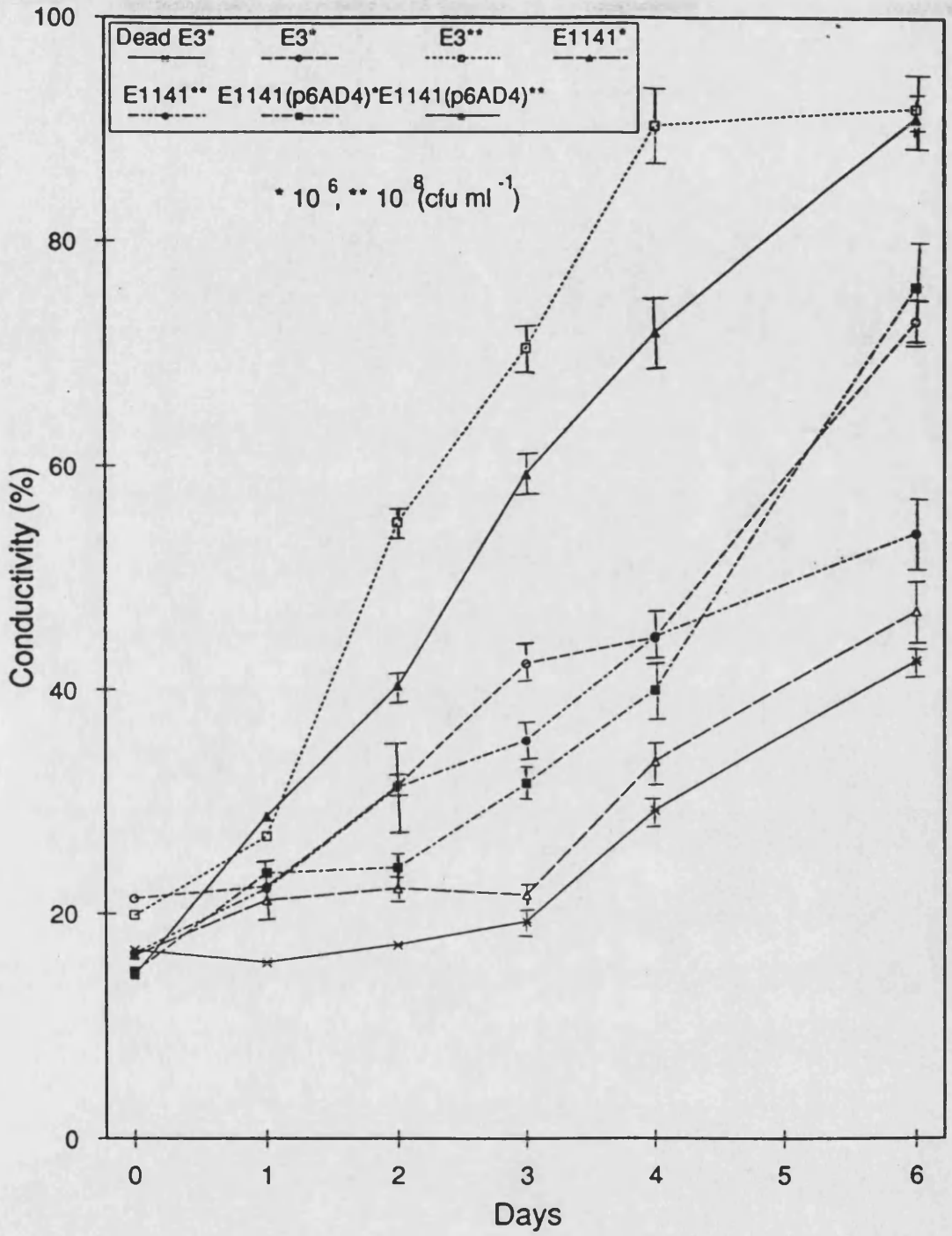


Figure 4.5: Conductivity of tomato cv. Rootstock leaves infiltrated with *X. campestris* pv. *vesicatoria* strians E3, E1141 and E114(p6AD4)

Leaves of tomato cv. Rootstock seedlings were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3 (wild type), E1141 (nonpathogenic mutant) and E1141(p6AD4) (pathogenic mutant) and incubated in a growth chamber at 25 °C, 89-92% rh, 16 h day and 180-250 µE m^{-2} s^{-1} light. Fifteen leaf discs (4mm diamter) were taken per replicate. Means of 5 replicates are shown with the standard error bars.

population of E1141 (non-pathogenic mutant) was fairly stable during the experimental period (Figure 4.6). The population growth of a low inoculum concentration of E3 increased 400 fold 6 days after infiltration, but the population of E1141(p6AD4) infiltrated at the same concentrations increased 200 fold 2 days after infiltration and then decreased (Figure 4.6). The limit of population growth of E3 and E1141(p6AD4) was lower in tomato cv. Rootstock than in pepper cv. ECW. The E1141 population decreased steadily when a high inoculum concentration (10^8 cfu ml^{-1}) was infiltrated in the leaves of tomato cv. Rootstock and reached the same level of its lower inoculum concentration (10^6 cfu ml^{-1}) 6 days after infiltration.

4.1.7 Interaction between Resistant Tomato cv Hawaii 7998 and *Xcv* Strains E3, E1141 and E1141(p6AD4)

Population growth, cell leakage and disease symptoms caused by infiltration with *Xcv* strains E3, E1141 and E1141(p6AD4) were assessed on seedlings of tomato cv. Hawaii 7998 infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of each strain. The data from five replicate seedlings per each inoculum concentration was transformed either to angular or log before it was analysed according to split plot design in which the plants and sampling times represented main blocks and subplots respectively.

4.1.7.1 Symptoms of Leaf Spot disease on Hawaii 7998 Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

Symptoms of leaf spot were scored according 1-6 scale (Lozano and Sequeira, 1970) where 1 represents no symptoms and 6 represents HR. Tomato cv. Hawaii 7998 leaves infiltrated with 10^8 cfu ml^{-1} of the pathogenic strains E3 and E1141(p6AD4) were the only leaves that developed an HR 2 to 3 days after infiltration (Table 4.5). Infiltrated areas become necrotic between 2-3 days after

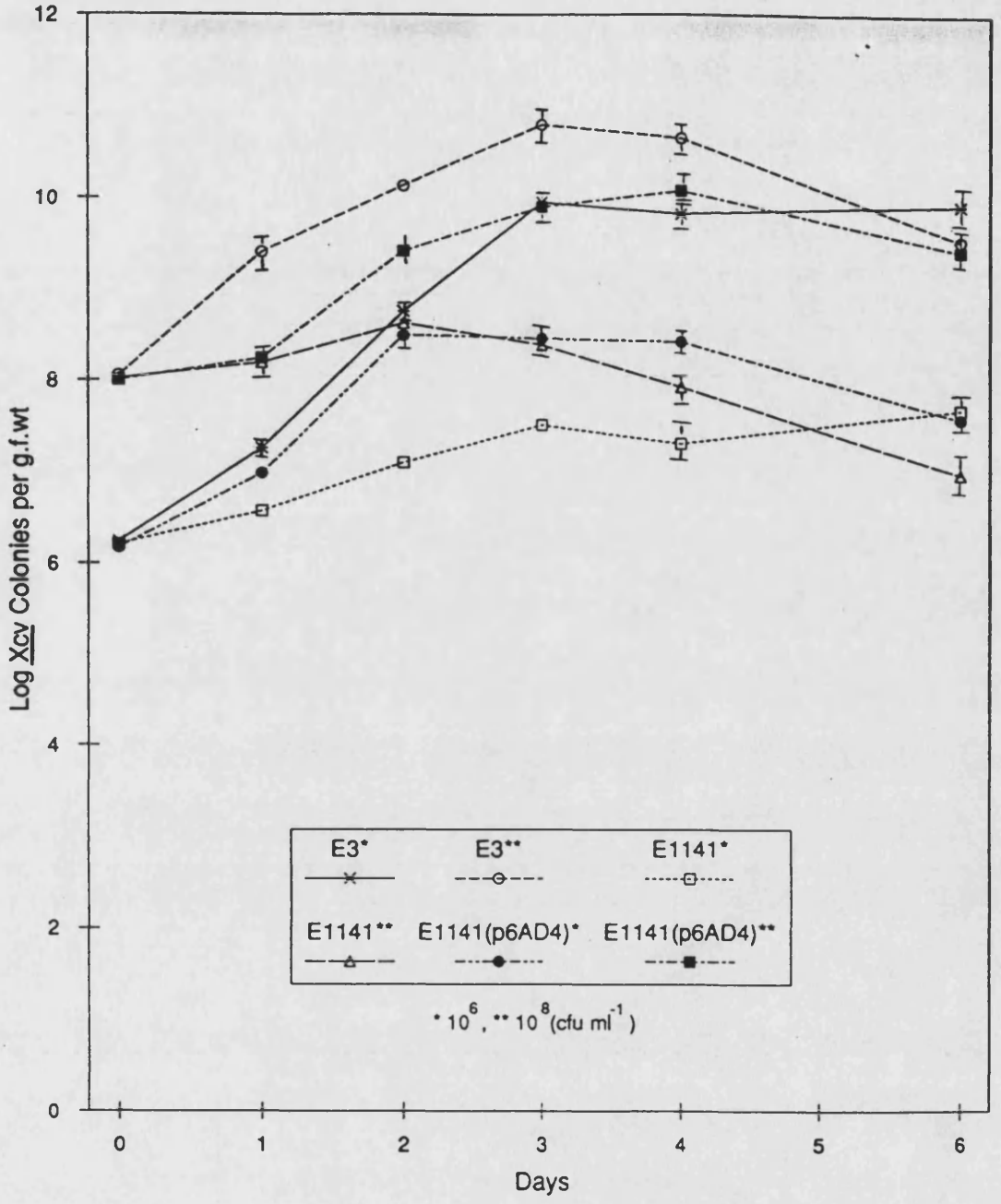


Figure 4.6: Comparative population trends of *X. campestris* pv. *vesicatoria* strians E3, E1141 and E114(p6AD4) infiltrated in leaves of tomato cv. Rootstock

Leaves of tomato cv. Rootstock seedlings were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3 (wild type), E1141 (nonpathogenic mutant) and E1141(p6AD4) (pathogenic mutant) and incubated in a growth chamber at 25 °C, 89-92% rh, 16 h day and 180-250 µE m^{-2} s^{-1} light. Fifteen leaf discs (4mm diamter) were taken per replicate. Means of 5 replicates are shown with the standard error bars.

Table 4.5: Leaf spot symptoms on resistant tomato (cv. Hawaii 7998) infiltrated with *Xcv* strains E3, E1141 and E1141(p6AD4)

Days	Treatments						
	Dead E3 (10^8)	E3 (10^6)	E3 (10^8)	E1141 (p6AD4) (10^6)	E1141 (p6AD4) (10^8)	E1141 (10^6)	E1141 (10^8)
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1	1.0	1.0	2.0	1.0	2.0	1.0	1.0
2	1.0	1.0	3.8 ± 0.20	1.0	3.6 ± 0.22	1.0	1.0
3	1.0	1.0	4.8 ± 0.18	1.0	4.6 ± 0.24	1.0	1.0
5	1.0	1.0	6.0	1.0	6.0	1.0	1.0
6	1.0	1.0	6.0	1.0	6.0	1.0	1.0

Seedlings of tomato cv. Hawii 7998 were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3, E1141 and E1141(p6AD4) and incubated at 25 ± 1 ^{0}C, 88-92% rh, 16 h photoperiod and light intensity of 180-250 μE m^{-2} s^{-1}. Plants were assessed for the development of disease symptoms almost every day using the scale of Lozano and Sequeira (1970) as follows:

1 = No symptoms; 2 = Yellowing; 3 = Small dark dots within the yellow infiltrated area; 4 = Small, necrotic, dark areas within a light brown infiltrated area; 5 = Entire infiltrated area light brown and surrounded by a dark halo; 6 = Typical hypersensitive reaction.

Data are the means and standard errors of 5 replicates for each treatment.

infiltration (Plate 4.6a). Lower inoculum concentrations (10^6 cfu ml^{-1}) of the pathogenic strains E3 did not induce an HR. Leaves of cv. Hawaii 7998 infiltrated with high and low inoculum concentrations of the non-pathogenic strain E1141 did not show an HR 6 days after infiltration (Plate 4.6b). Symptoms varied significantly ($p < 0.001$) between leaves of cv. Hawaii 7998 infiltrated with high and low inoculum concentrations of *Xcv* strains 6 days after infiltration (Appendix 9). Symptoms induction was also significant ($p < 0.01$) with time and the interaction between *Xcv* inocula and time was also significant ($p < 0.05$) (Appendix 9).

4.1.7.2 Conductivity of the Leaves of Resistant Tomato cv. Hawaii 7998 Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

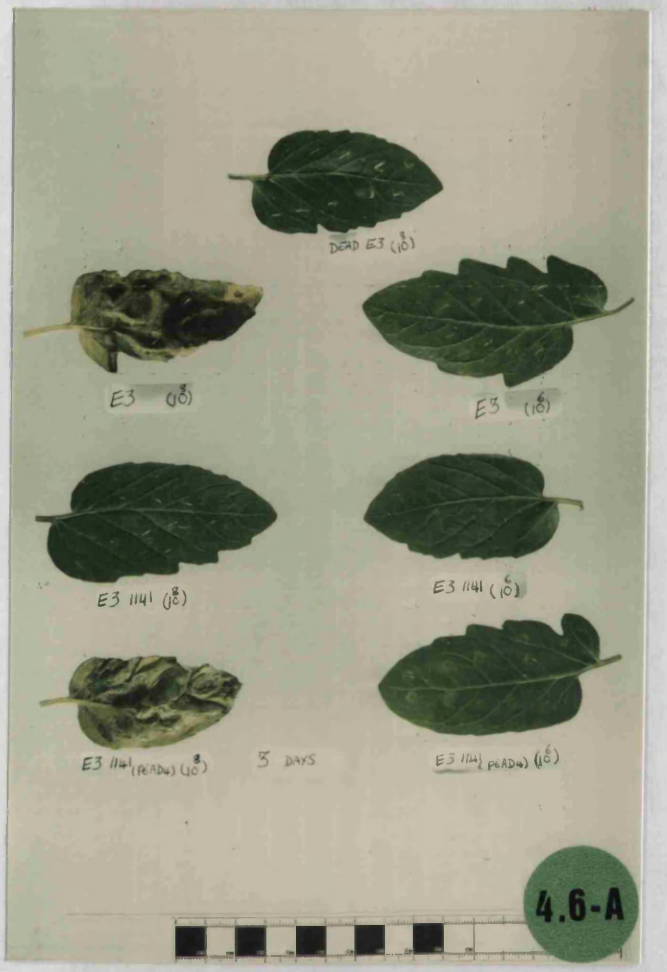
Cell leakage of tomato cv. Hawaii 7998 leaves varied significantly with the inoculum concentration of *Xcv* strain, and time after infiltration (Appendix 10). The interaction between inoculum concentration and time was also significant. Cell leakage of cv. Hawaii 7998 leaves infiltrated with 10^8 cfu ml^{-1} of the pathogenic strains E3 and E1141(p6AD4) increased rapidly within 48 hour due to an HR and the conductivity of E3 was higher than E1141(p6AD4) (Figure 4.7). The cell leakage caused by the high inoculum concentration (10^8 cfu ml^{-1}) of the non-pathogenic strain E1141 and the low inoculum concentration (10^6 cfu ml^{-1}) of all *Xcv* strains was similar to that caused by the infiltration with dead E3 cells (Figure 4.7).

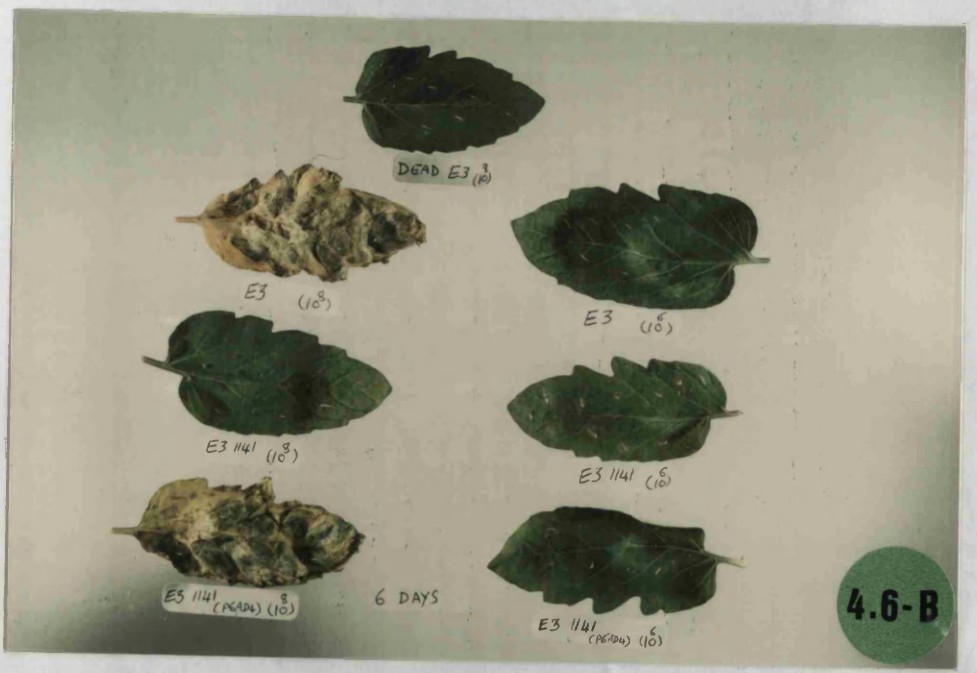
4.1.7.3 Population Growth of *Xcv* Strains E3, E1141 and E1141(p6AD4) in the Leaves of Resistant Tomato Hawaii 7998

The population changes were significant ($p < 0.001$) with the inoculum concentrations of *Xcv* strains (Appendix 11). The population of all *Xcv* strains increased initially in leaves of Hawaii 7998 infiltrated with high and low

Plate 4.6a: Symptoms of leaf spot disease on tomato (cv. Hawii 7998) leaves 3 days after infiltration with 10^6 and 10^8 cfu ml^{-1} of E3, E141 and E1141[p6AD4].

Plate 4.6b: Symptoms of leaf spot disease on tomato (cv. Hawii 7998) leaves 6 days after infiltration with 10^6 and 10^8 cfu ml^{-1} of E3, E141 and E1141[p6AD4].


DEAD E3 (10^8)
E3 (10^8)
E3 (10^6)
E3 1141 (10^8)
E3 1141 (10^6)
E3 1141 (PCADH) (10^8)
3 DAYS
E3 1141 (PCADH) (10^6)
4.6-A


DEAD E3 (10^8)
E3 (10^8)
E3 (10^6)
E3 1141 (10^8)
E3 1141 (10^6)
E3 1141 (PCADH) (10^8)
6 DAYS
E3 1141 (PCADH) (10^6)
4.6-B

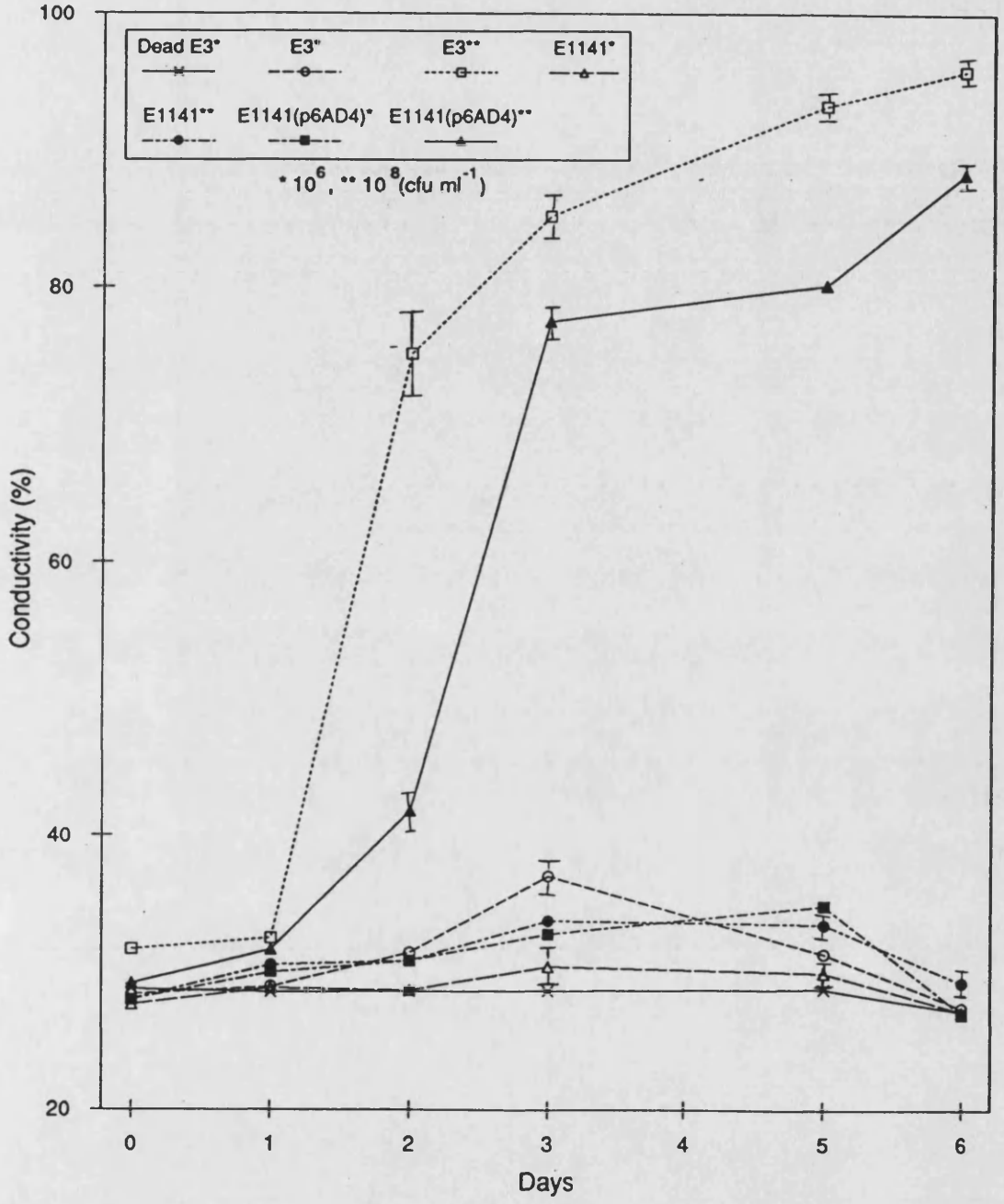


Figure 4.7: Conductivity of tomato cv. Hawaii 7998 leaves infiltrated with *X. campestris* pv. *vesicatoria* strians E3, E1141 and E114(p6AD4)

Leaves of tomato cv. Hawaii 7998 seedlings were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3 (wild type), E1141 (nonpathogenic mutant) and E1141(p6AD4) (pathogenic mutant) and incubated in a growth chamber at 25 °C, 89-92% rh, 16 h day and 180-250 µE m^{-2} s^{-1} light. Fifteen leaf discs (4mm diamter) were taken per replicate. Means of 5 replicates are shown with the standard error bars.

inoculum concentrations regardless of their pathogenicity (Figure 4.8). The population of the pathogenic strains E3 and E1141(p6AD4), decreased steadily when infiltrated at higher inoculum concentrations (10^8 cfu ml^{-1}) and an HR was induced 2 days after infiltration (Figure 4.8). The population of non-pathogenic strain of *Xcv* E1141 increased steadily in leaves infiltrated with 10^8 cfu ml^{-1} reaching the highest level of all the strains, but was unable to induce an HR 6 day after infiltration. Population changes in cv. Hawaii 7998 leaves infiltrated with 10^6 cfu ml^{-1} of the three *Xcv* strains followed similar patterns as those of the high inoculum concentrations (10^8 cfu ml^{-1}). The non-pathogenic strain E1141 also had the highest multiplication rate and reached the same population level of the high inoculum concentration 6 days after infiltration (Figure 4.8). The interaction between concentration and time was also significant ($p < 0.001$) (Appendix 11) which means that the multiplication of each strain was affected by the initial concentration of the inoculum and the time after infiltration. The population of E3 and E1141(p6AD4) when infiltrated in a low concentration did not increase to a level which was able to induce a visible HR in the tomato cv. Hawaii 7998 leaves (Figure 4.8)

4.2 Interaction Between *Xcv* Strains and Cells of Tomato cv. Rootstock *in vitro*

This study was conducted to investigate the reaction of tomato cv. Rootstock cells from different phases of suspension cultures with different *Xcv* strains *in vitro*. The possible production of a low molecular weight toxin(s) in the co-culture medium of *Xcv* strain (E3) with tomato cells was also investigated.

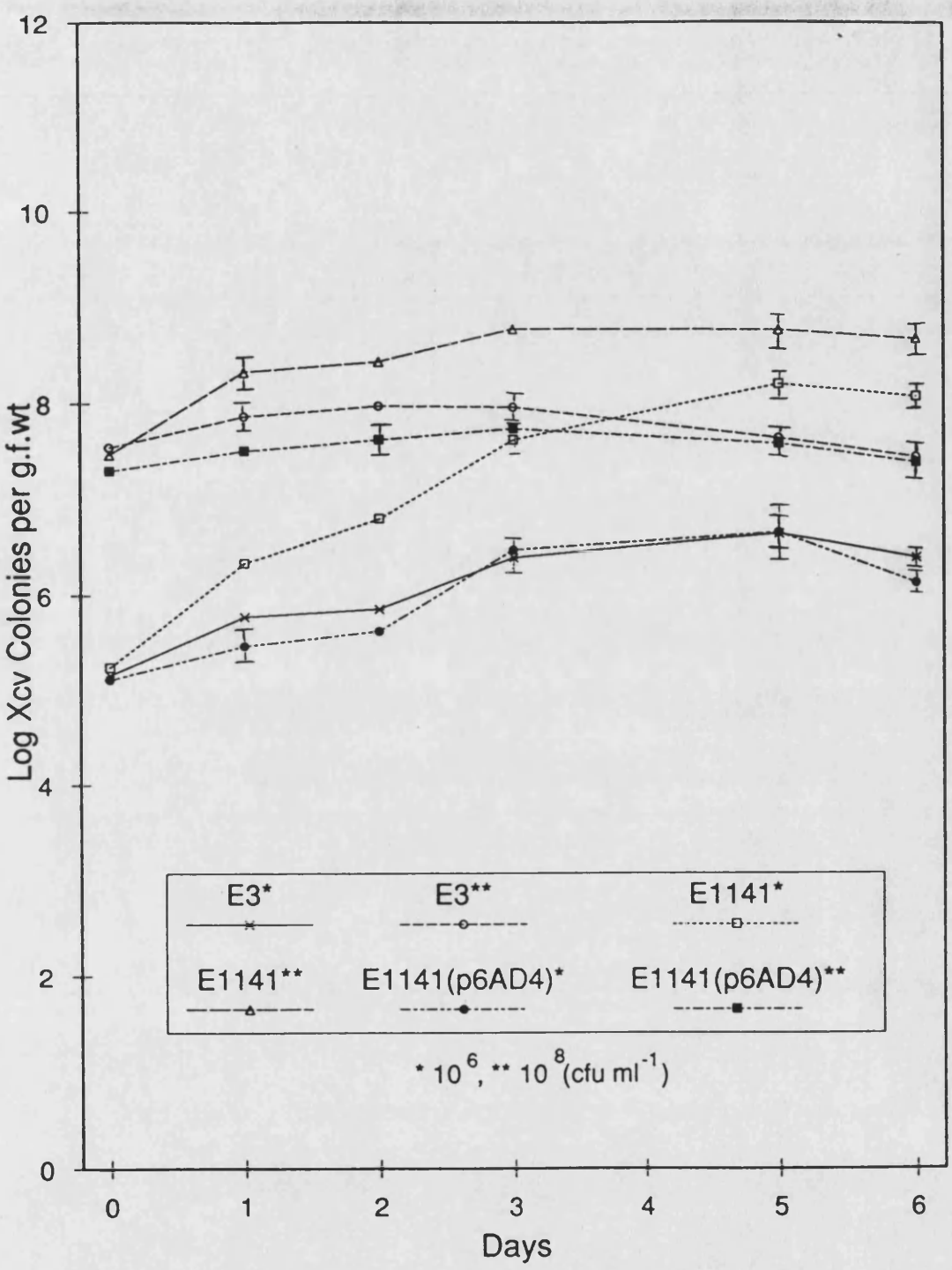


Figure 4.8: Comparative population trends of *X. campestris* pv. *vesicatoria* strians E3, E1141 and E114(p6AD4) infiltrated in leaves of tomato cv. Hawaii 7998

Leaves of tomato cv. Hawaii 7998 seedlings were infiltrated with two inoculum concentrations (10^6 and 10^8 cfu ml^{-1}) of *Xcv* strains E3 (wild type), E1141 (nonpathogenic mutant) and E1141(p6AD4) (pathogenic mutant) and incubated in a growth chamber at 25 °C, 89-92% rh, 16 h day and 180-250 µE m^{-2} s^{-1} light. Fifteen leaf discs (4mm diamter) were taken per replicate. Means of 5 replicates are shown with the standard error bars.

4.2.1 Materials and Methods

Unless otherwise stated, the preparation of *Xcv* inocula from overnight cultures was as described in Section 2.5.3, and 10 ml of each inoculum (10^8 cfu ml^{-1}) were added to 90 ml of either distilled water or 5% pcv of suspension of cells of tomato cv. Rootctock in distilled water prepared as described in Section 2.3.5. The final concentration of the bacterial cells in the suspension was approximately 10^7 cfu ml^{-1}. The viability of the bacterial cells was assessed by the plating described in Section 2.5.6, and the viability of the tomato cells were assessed using FDA as described in Sections 2.3.5. Three replications of each treatment were incubated in an orbital shaker described in Section 2.3.5.

4.2.2 Growth Rate of Tomato Suspension Cultures

The growth rate of a tomato suspension was investigated to determine the different phases. Suspension cultures giving final concentrations of 5% pcv were initiated in 25 ml of B5 medium and the fresh weight of these tomato suspension cells was determined every 2 days in 4 replicate flasks. The contents of these flasks were transferred to syringes of known weights and the excess medium was removed through a filter in each syringe. Then the syringes were spun at 3000 g for further removal of the B5 medium. The difference in fresh weight represented cell growth. The growth phases of the tomato cell suspension were lag, exponential and stationary (Figure 4.9). the lag phase lasted for 6 days after which the exponential phase started, and following this the doubling time on the exponential growth phase was 10 days. The stationary phase started after 18 days.

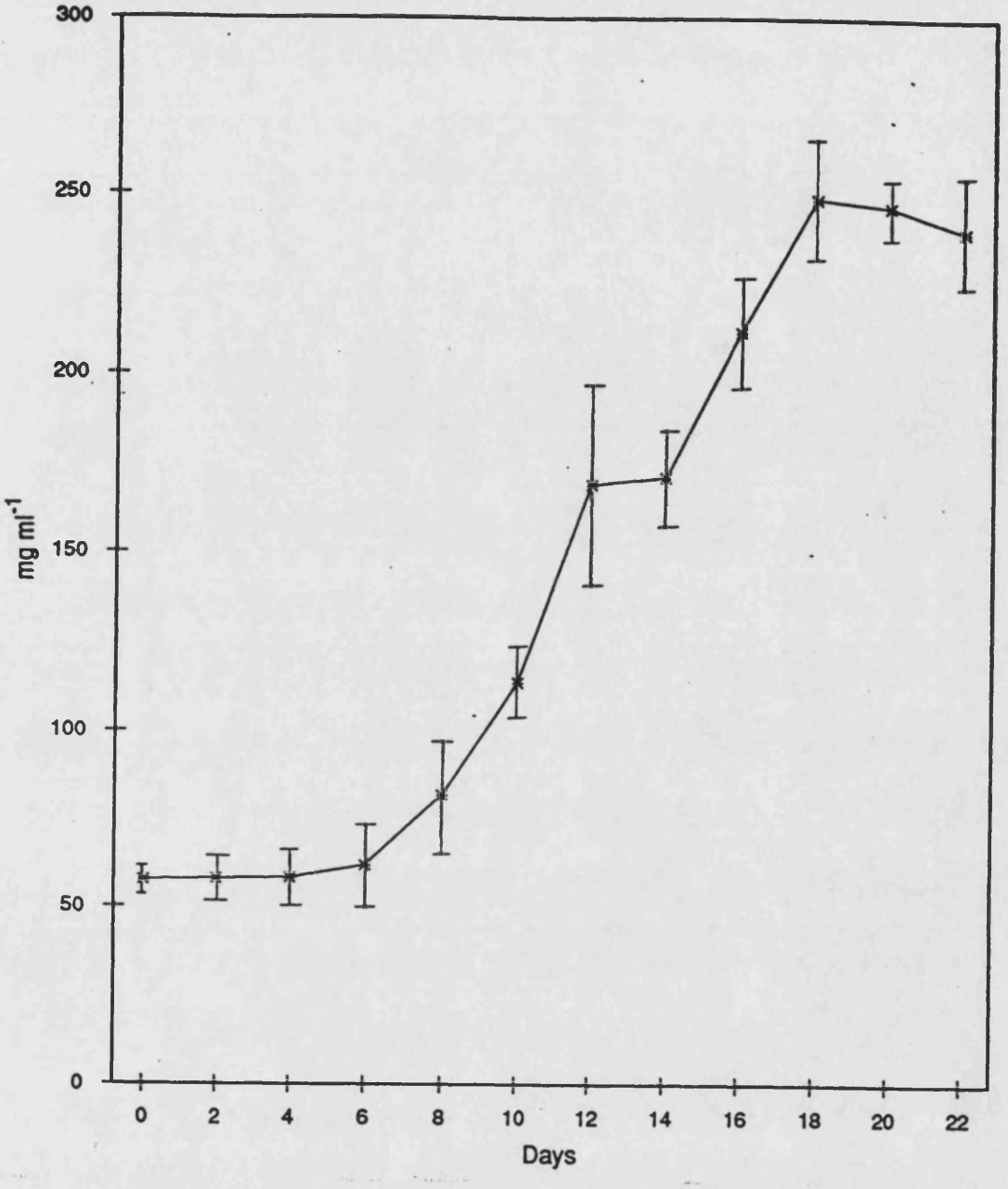


Figure 4.9 Growth of tomato cv. Rootstock in cell suspension cultures

Suspension cultures of 5% pcv were initiated in 25 ml B5 medium and incubated in an orbital shaker incubator 120 rpm, at 25 ± 1 °C, 16 h photoperiod and light intensity of 150 μE m^{-2} s^{-1}. Flasks were taken every two days, transferred to syringes of known weights and the excess media was removed through filters. These syringes were spun at 3000 g for futher removal of the medium. The difference in weights represented the growth (fresh weight) of the cell suspension. Means of 4 replicates are shown with the standard error bars.

4.2.3 Determination of the Optimum Medium for the Study of the Interaction between *Xcv* Strains and Tomato Suspension Cells

Media used to study the interaction between host and pathogen *in vitro* should allow both to survive, but not favour one over the other. The media tested for this were distilled water and dilutions of B5 medium $\frac{1}{10}$B5, $\frac{1}{5}$B5, $\frac{1}{2}$B5 and B5. Three replicates of each medium were cultured with either 10^7 cfu ml^{-1} of E3 or 5% pcv suspensions from exponential phase and the survival of bacterial and tomato cells were assessed. The split plot design and partial testing were used for analysis of tomato cell survival in which flask and sampling time represented the block and subplot respectively.

There was no difference between the percentage of tomato cells which survived in distilled water compared to the survival of cells in the dilutions of B5 medium (Appendix 12), but the survival of tomato cells decreased significantly with the length of time in all the media ($p < 0.001$). The means of distilled water and the overall means of B5 dilutions are presented in Figure (4.10).

The population of *Xcv* strain E3 in distilled water and $\frac{1}{10}$B5 increased in the first and second day and then started to decrease with time (Table 4.6). In $\frac{1}{5}$B5 the population increased 24 h after cultures initiation and then decreased with time. No bacteria survived in $\frac{1}{5}$B5 4 days after cultures initiation (Table 4.6). The full strength of B5 salts was not suitable for the survival of *Xcv* E3 and the numbers were substantially reduced 2 hours after infiltration in this medium. From this experiment, distilled water and $\frac{1}{10}$B5 were the best media for the survival of both bacteria and tomato cells. Distilled water was selected for further studies of cell killing ability of E3 because it has no nutrients that can affect the interaction.

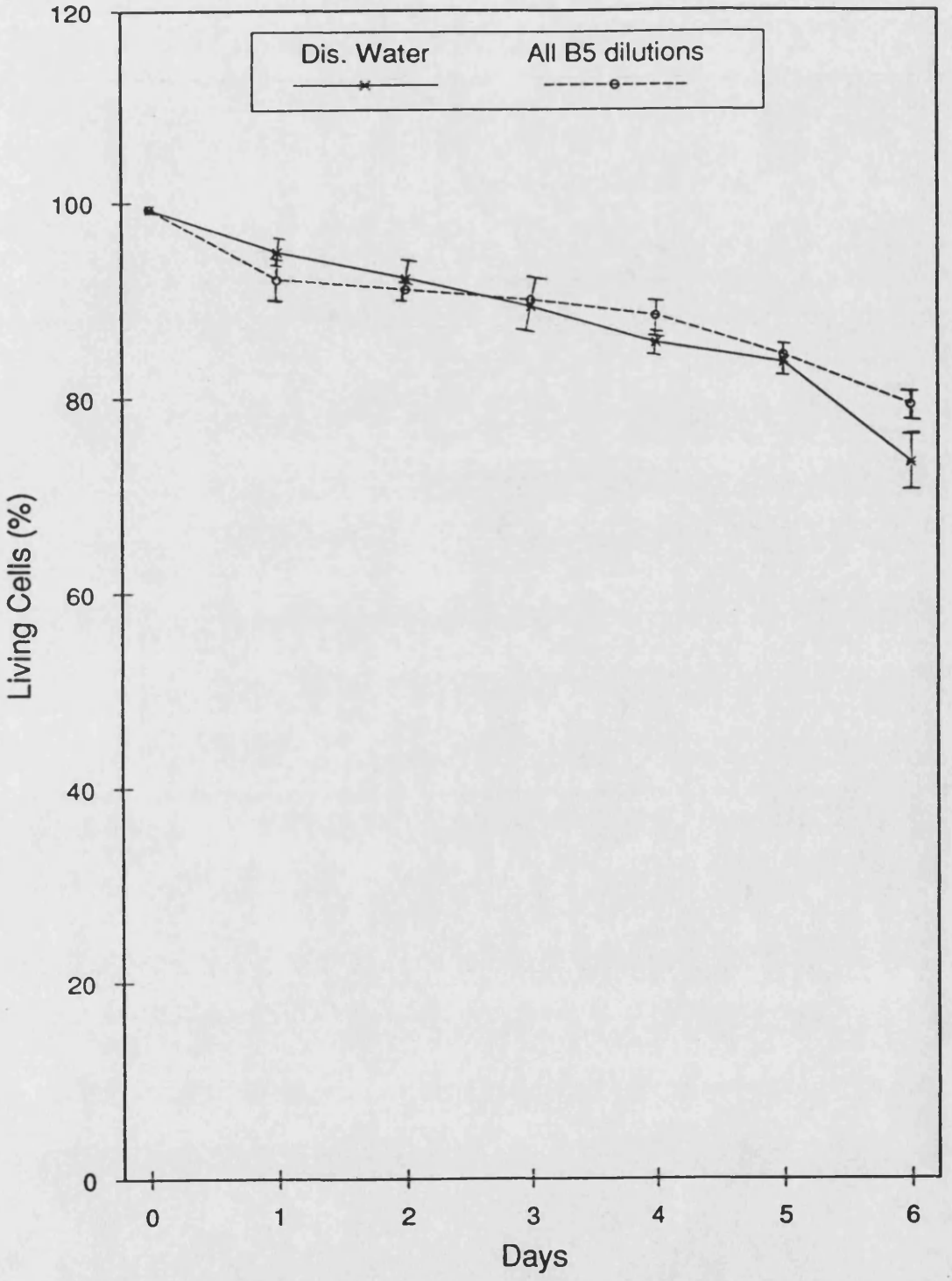


Figure 4.10 : Survival of cells of tomato cv. Rootstock cells in distilled water and dilutions of B5 medium

Media used for the investigation for bacterial survival were Distilled water, 0.1 B5, 0.2 B5, 0.5 B5 and B5. Three flasks of each medium were subcultured with 5% pcv suspension of tomato cv. Rootstock cells from exponential phase and incubated in an orbital shaker incubator 120 rpm, 16 hours photoperiod and light intensity was 150 $\mu E\ m^{-2}\ s^{-1}$. Means of 3 replicates are shown with the standard error bars.

Table 4.6: Effect of different media on the survival of *Xcv* strain E3

days	Mean number of E3 (10^5 cfu ml^{-1})				
	D. Water	$\frac{1}{10}$B5	$\frac{1}{5}$B5	$\frac{1}{2}$B5	B5
0	90 ± 10	94 ± 13	77 ± 4	53 ± 1	17 ± 4
1	115 ± 32	222 ± 25	192 ± 17	5 ± 1	3 ± 1
2	117 ± 11	175 ± 20	113 ± 5	5 ± 1	1 ± 0
3	97 ± 3	32 ± 1	27 ± 1	2 ± 1	0
4	77 ± 4	25 ± 2	5 ± 0.6	2 ± 1	0
5	14 ± 2	1 ± 0.1	0	0	0
6	0	2 ± 0.4	0	0	0

The concentration of E3 inoculum in each of the media was 10^7 cfu ml^{-1} at the start of the experiment. The cultures were incubated in an orbital shaker 120 rpm at 25 ± 1 ^{0}C, 16 h photoperiod and light intensity of 150 μE m^{-2} s^{-1}. Samples were taken after two hours on 0 day.

Data are the means and standard errors of 3 replicates for each treatment.

In a preliminary experiment testing the killing ability of E3 on tomato cells,the survival of E3 was severely affected after 24 h in distilled water (the control) possibly due to a change in pH. The survival of E3 and tomato cells was assessed in a buffer (MES) at different concentrations, with the pH was adjusted to 5.8. The best survival of tomato cells was found in 1.0 mM MES and distilled water, but the percentage of living tomato cells decreased with increasing concentration of MES (Figure 4.11). *Xcv* E3 survival was equally good in all MES concentrations (Table 4.7). From these results 1.0 mM MES was chosen as the best medium for the assessment of the cell killing ability of E3.

4.2.4 Bioassay of Cell Killing Ability of Different *Xcv* Strains on Cells of Tomato (cv. Rootstock) from Different Phases of Suspension Growth

The killing ability of *Xcv* strains E3, E1141, E1141(p6AD4) was assessed on tomato cells from different phases of cell suspension (lag, exponential, and stationary). Three replicates of co-culture between tomato cells (5% pcv) from suspension culture and each *Xcv* strain (10^7 cfu ml^{-1}) performed as in Section (4.2.1). Control treatments of each *Xcv* strain (10^7 cfu ml^{-1}) without tomato cells and a control treatment of tomato cells (5% pcv) without bacteria were included.

4.2.5 Bioassay of Tomato Cells from Lag Phase with *Xcv* Strains

Three *Xcv* strains E3, E1141 and E1141(p6AD4) were bioassayed with cells (5% pcv) from lag phase (5 day-old). The percentage of living tomato cells decreased when co-cultured with all *Xcv* strains irrespective of the strain pathogenicity (Figure 4.12) and this decrease was significantly different with the time in all co-culture media including the control ($p < 0.001$, Appendix 13a). When the effect of distilled water was omitted from the statistical analysis, the survival of the tomato cells was also affected significantly with the type of *Xcv* strain and the time of co-culture ($p < 0.001$, Appendix 13b). The interaction

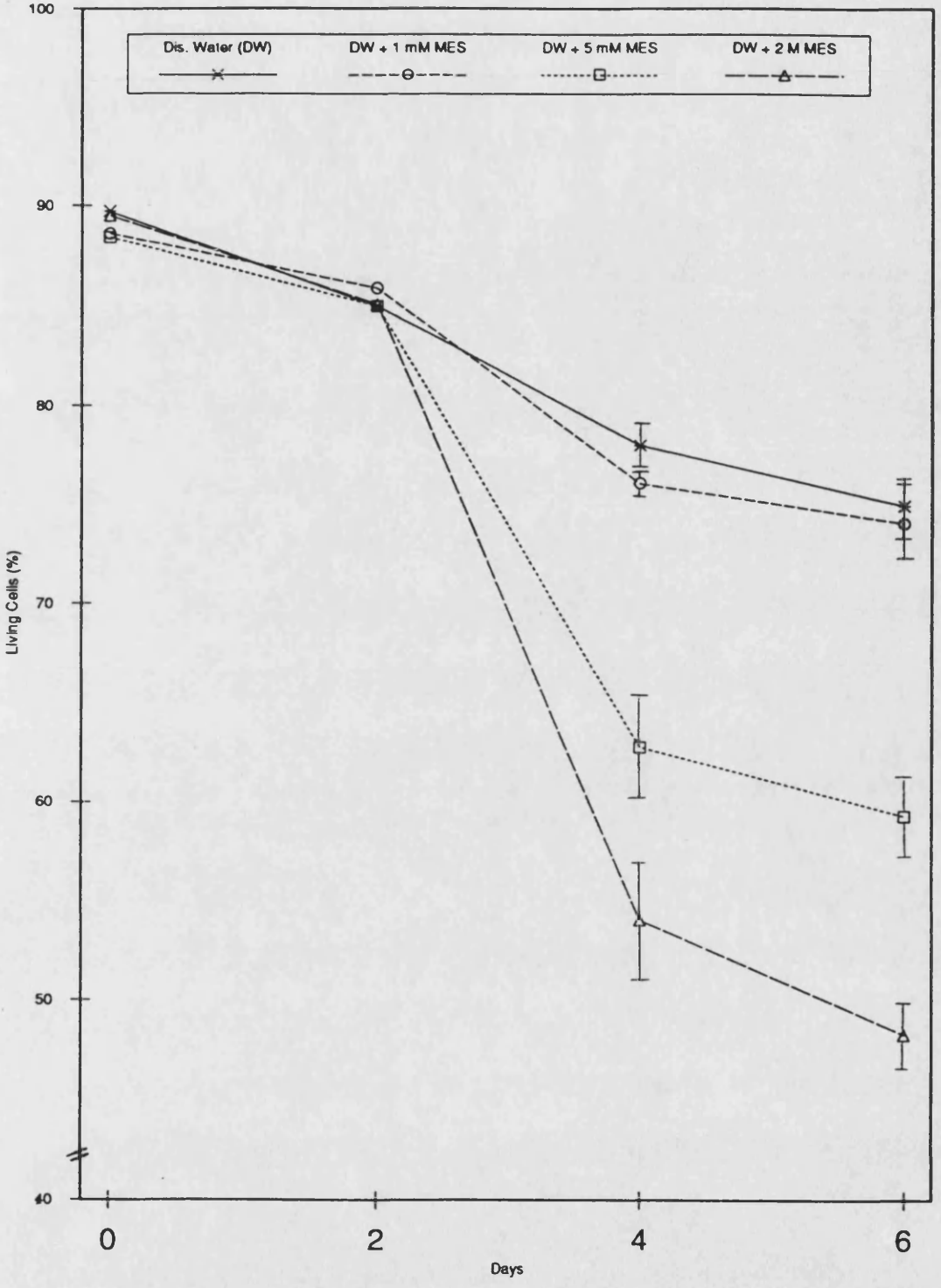


Figure 4.11: survival cells of tomato cv. Rootstock in MES

Different concentrations of the buffer MES (0, 1.0 mM, 5.0 mM and 2.0 M) were used and the pH was adjusted to 5.8 before autoclaving. Suspension cultures (5% pcv) of tomato cv. Rootstock cells were initiated from 10 day-old cultures and incubated in an orbital shaker incubator 120 rpm, 16 hours photoperiod and light intensity was 150 $\mu E\ m^{-2}\ s^{-1}$. Means of 3 replicates are shown with the standard error bars.

Table 4.7: Number (cfu ml^{-1}) of *Xcv* strain E3 in MES media

Days	Medium			
	Distilled Water	1 mM MES	2 mM MES	2 M MES
0	3.2×10^7	3.3×10^7	3.0×10^7	2.9×10^7
2	1.7×10^7	1.9×10^7	1.9×10^7	1.8×10^7
4	5.0×10^5	5.7×10^5	1.6×10^6	2.0×10^5
6	4.4×10^5	4.7×10^5	4.7×10^5	1.3×10^5

The concentration of E3 inoculum in each of the media was 10^7 cfu ml^{-1} at the start of the experiment. The cultures were incubated in an orbital shaker 120 rpm at 25 ± 1 ^{0}C, 16 h photoperiod and light intensity of 150 µE m^{-2} s^{-1}. Samples were taken after two hours on day 0.

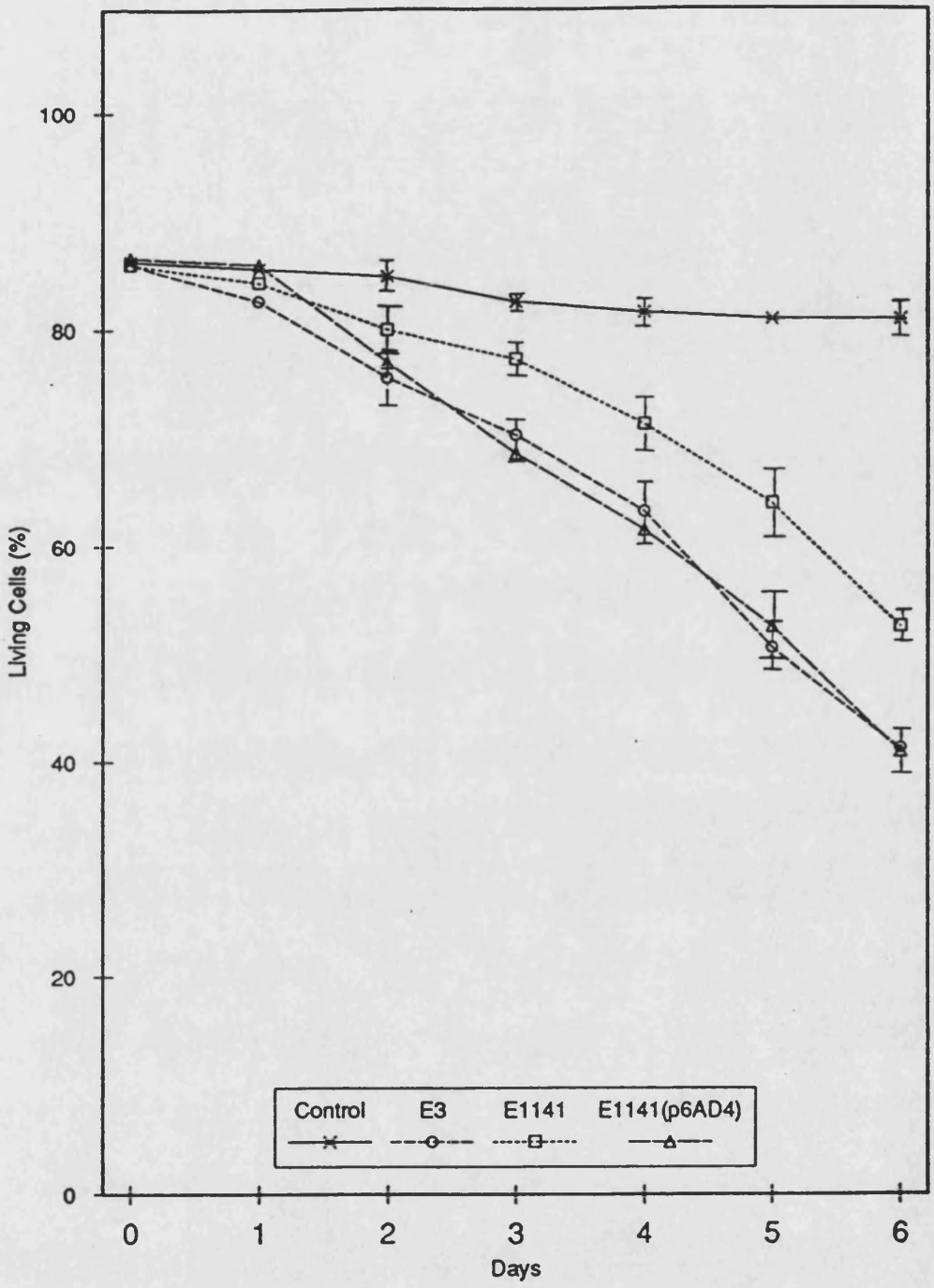


Figure 4.12: Bioassay of cell killing ability of *X. campestris* pv. *vesicatoria* strains E3, E1141, and E1141(p6AD4) on cells of tomato cv. Rootstock from lag phase

10 ml of *Xcv* strains E3, E1141 and E1141(p6AD4) inocula (10^7 cfu ml^{-1}) were added to 5% pcv suspension cultures of tomato cv. Rootstock cells initiated from 5 days-old suspension cultures and incubated in an orbital shaker incubator 120 rpm, 16 hours photoperiod and light intensity was 150 µE m^{-2} s^{-1}. Means of 3 replicates are shown with the standard error bars.

between each *Xcv* strain and the time was also significantly different ($p < 0.05$, Appendix 13b)). The non-pathogenic *Xcv* strain E1141 caused less cell killing with time compared to the pathogenic strains E3 and E1141(p6AD4) (Figure 4.12). When the non-pahtogenic *Xcv* strain E1141 was omitted from the statistical analysis,no difference between the pathogenic strains E3 and E1141(p6AD4) in their cell killing ability or their interaction with time was found, but the effect of time was significantly different ($p < 0.001$, Appendix 13c). This indicated that the pathogenic strains have significantly higher cell killing ability than E1141 although the presence of any strain in the media could cause a considerable killing of tomato cells compared to that of the control (Figure 4.12). All bacterial strains multiplied in the presence of tomato cells and increased 200-300 fold, but those which were kept in the distilled water decreased with time by 200-300 fold (Table 4.8).

4.2.6 Bioassay of Tomato Cells from Exponential Phase with *Xcv* Strains

E3, E1141 and E1141(p6AD4) were co-cultured with cells of tomato cv. Rootstock from exponential phase to test their cell killing ability. The survival of tomato cells decreased with time (Figure 4.13), and the effect of the time on the cells survival was significantly different ($p < 0.001$, Appendix 14a). The percentage of living cells was reduced in the different treatments, but the bacterial strains had higher killing ability compared to the control (Figure 4.13). When the survival of tomato cells in the control was omitted from the the statistical analysis, the killing ability of *Xcv* strains was significantly different ($p < 0.001$) and the effect of these strais vary significantly when the time of interaction increased ($p < 0.001$, Appendix 14b). The pathogenic *Xcv* strains E3 and E1141(p6AD4) had a higher tomato cell killing ability compared to E1141 (Figure 4.13). E3 had a significantly higher killing ability than E1141(p6AD4) ($p < 0.001$, Appendix 14c), but the interaction between the two strains and time was not

Table 4.8: Number of *Xcv* strains E3, E1141 and E1141(p6AD4) survived in tomato cells (lag phase) killing ability bioassay

Days	E3		E1141		$E1141_{(p6AD4)}$	
	With Tomato Cells	Control	With Tomato Cells	Control	With Tomato Cells	Control
0	1.1×10^7	1.2×10^7	1.1×10^7	1.0×10^7	1.3×10^7	1.3×10^7
1	1.0×10^8	9.3×10^6	8.3×10^7	9.7×10^6	7.7×10^6	7.0×10^6
2	1.5×10^8	6.7×10^5	1.6×10^8	5.3×10^5	3.7×10^6	5.1×10^6
3	2.3×10^8	4.6×10^5	2.2×10^8	4.2×10^5	1.2×10^7	3.7×10^6
4	3.7×10^8	2.8×10^5	3.3×10^8	3.0×10^5	6.7×10^8	5.0×10^5
5	1.0×10^9	1.2×10^5	5.7×10^8	1.4×10^5	8.7×10^8	3.3×10^5
6	1.2×10^9	1.1×10^5	6.7×10^8	6.0×10^4	7.3×10^8	2.0×10^4

The inoculum concentration of *Xcv* strains in 1.0 mM MES (pH 5.8) was 10^7 cfu ml^{-1} at the start of the experiment. The cultures were incubated in an orbital shaker 120 rpm at 25 ± 1 ^{0}C, 16 h photoperiod and light intensity of 150 μE m^{-2} s^{-1}. Samples were taken after two hours on day 0.

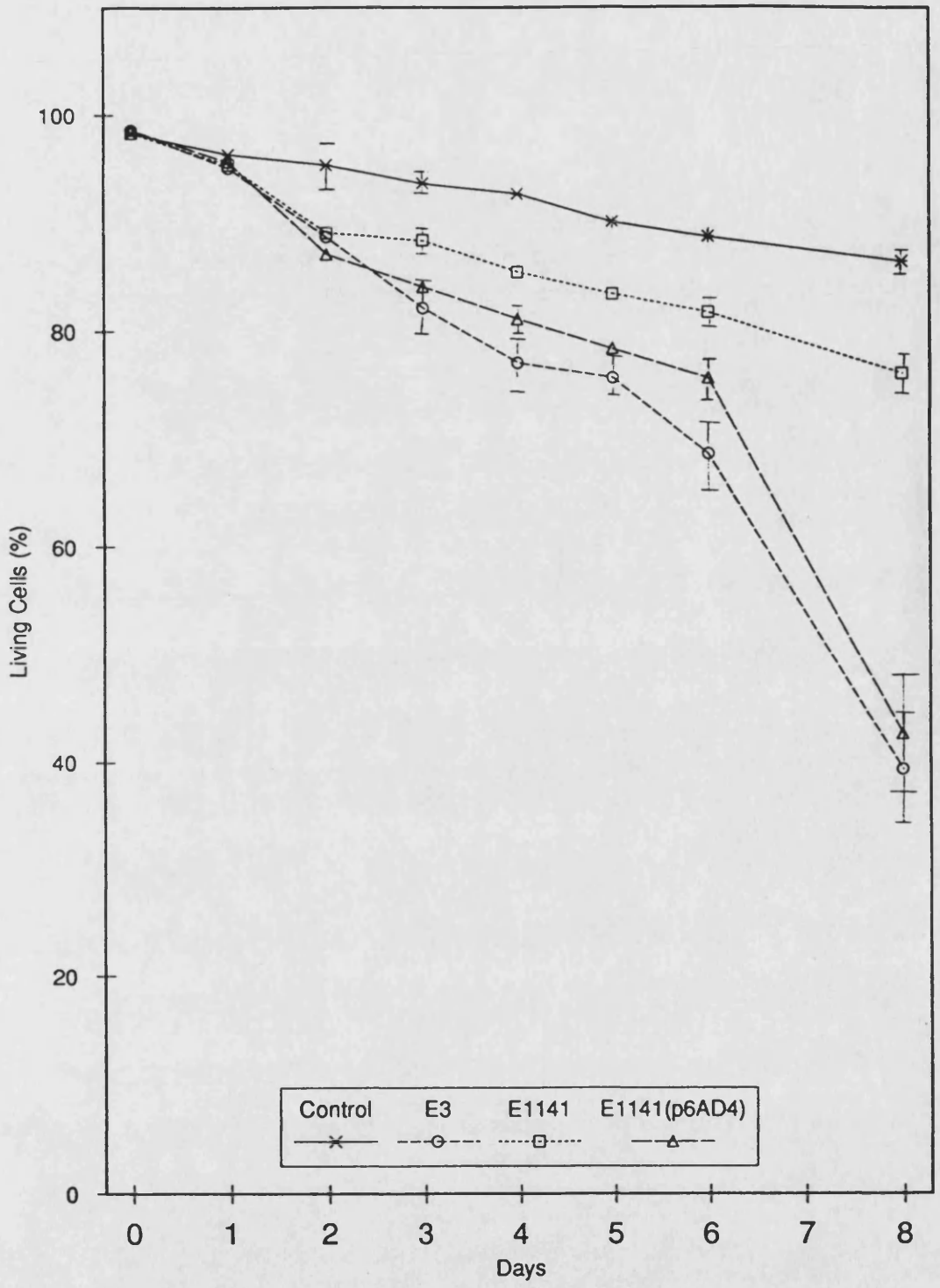


Figure 4.13: Bioassay of cell killing ability of *X. campestris* pv. *vesicatoria* strains E3, E1141, and E1141(p6AD4) on cells of tomato cv. Rootstock from exponential phase

10 ml of *Xcv* strains E3, E1141 and E1141(p6AD4) inocula (10^7 cfu ml^{-1}) were added to 5% pcv suspension cultures of tomato cv. Rootstock cells initiated from 10 day-old suspension cultures and incubated in an orbital shaker incubator 120 rpm, 16 hours photoperiod and light intensity was 150 µE m^{-2} s^{-1}. Means of 3 replicates are shown with the standard error bars.

significantly different. The length of time taken by these pathogenic bacterial strains to cause the death of the tomato cells was longer for the exponential phase cells (8 days) than the lag phase cells (6 days). The bacterial population increased when co-cultured with tomato cells. The E3 population increased until the fourth day and then started to fall (Table 4.9). E1141 and E1141(p6AD4) numbers fluctuated from day to day but higher than the numbers in the control.

4.2.7 Bioassay of Tomato Cells from Stationary Phase with *Xcv* Strains

Tomato cells from the stationary phase (18 days) were bioassayed with *Xcv* strains E3, E1141 and E1141(p6AD4). The percentage of living tomato cells at the start of the experiment was less compared to the other phases (lag and log). No tomato cell survived more than 4 days when co-cultured with all *Xcv* strains, while 75.6% of the cells were alive in the control (Figure 4.14). The results of the first 3 days were analysed according to the split plot design and partially tested. The *Xcv* strains a had significant ($p < 0.001$) effect on the survival of the tomato cells as did the length of time and the interaction between time and the different strains including the control ($p < 0.001$, Appendix 15a). When the control was omitted from the statistical analysis, survival of tomato cells varied significantly ($p < 0.001$) when co-cultured with different *Xcv* strains (Appendix 15b). The length of co-culture time with each strain also had a significant ($p < 0.001$) on the survival of cells. The pathogenic strains (E3 and E1141(p6AD4)) were found to be significantly different ($p < 0.05$) in their cell killing ability 3 days after co-culture (Appendix 15c) as well as the effect of time ($p < 0.001$, Appendix 15c) and the interaction between time and the strains of *Xcv* ($p < 0.01$, Appendix 15c). The number of bacterial cells of all strains increased with time when co-cultured with tomato cells. However, those in the control decreased in their numbers with time (Table 4.10).

Table 4.9: Number of *Xcv* strains E3, E1141 and E1141(p6AD4) survived in tomato cells (exponential phase) killing ability bioassay

Days	E3		E1141		$E1141_{(p6AD4)}$	
	With Tomato Cells	Control	With Tomato Cells	Control	With Tomato Cells	Control
0	1.7×10^7	1.2×10^7	1.5×10^7	1.4×10^7	1.6×10^7	1.3×10^7
1	2.7×10^7	1.4×10^7	3.0×10^7	3.0×10^7	9.3×10^6	5.0×10^6
2	3.0×10^7	7.8×10^6	2.1×10^7	1.2×10^7	2.0×10^7	5.1×10^6
3	3.7×10^7	4.7×10^6	2.7×10^7	3.0×10^6	2.4×10^7	3.0×10^6
4	2.6×10^8	5.3×10^5	1.4×10^7	1.8×10^6	1.5×10^6	3.0×10^5
5	6.5×10^7	4.0×10^5	9.3×10^7	5.9×10^5	2.2×10^7	4.3×10^5
6	6.3×10^7	3.0×10^5	4.5×10^7	2.7×10^5	1.3×10^7	2.7×10^5
8	5.7×10^7	2.6×10^5	4.5×10^7	1.8×10^5	1.4×10^7	9.3×10^4

The inoculum concentration of *Xcv* strains in 1.0 mM MES (pH 5.8) was 10^7 cfu ml^{-1} at the start of the experiment. The cultures were incubated in an orbital shaker 120 rpm at 25 ± 1 ^{0}C, 16 h photoperiod and light intensity of 150 μE m^{-2} s^{-1}. Samples were taken after two hours on day 0.

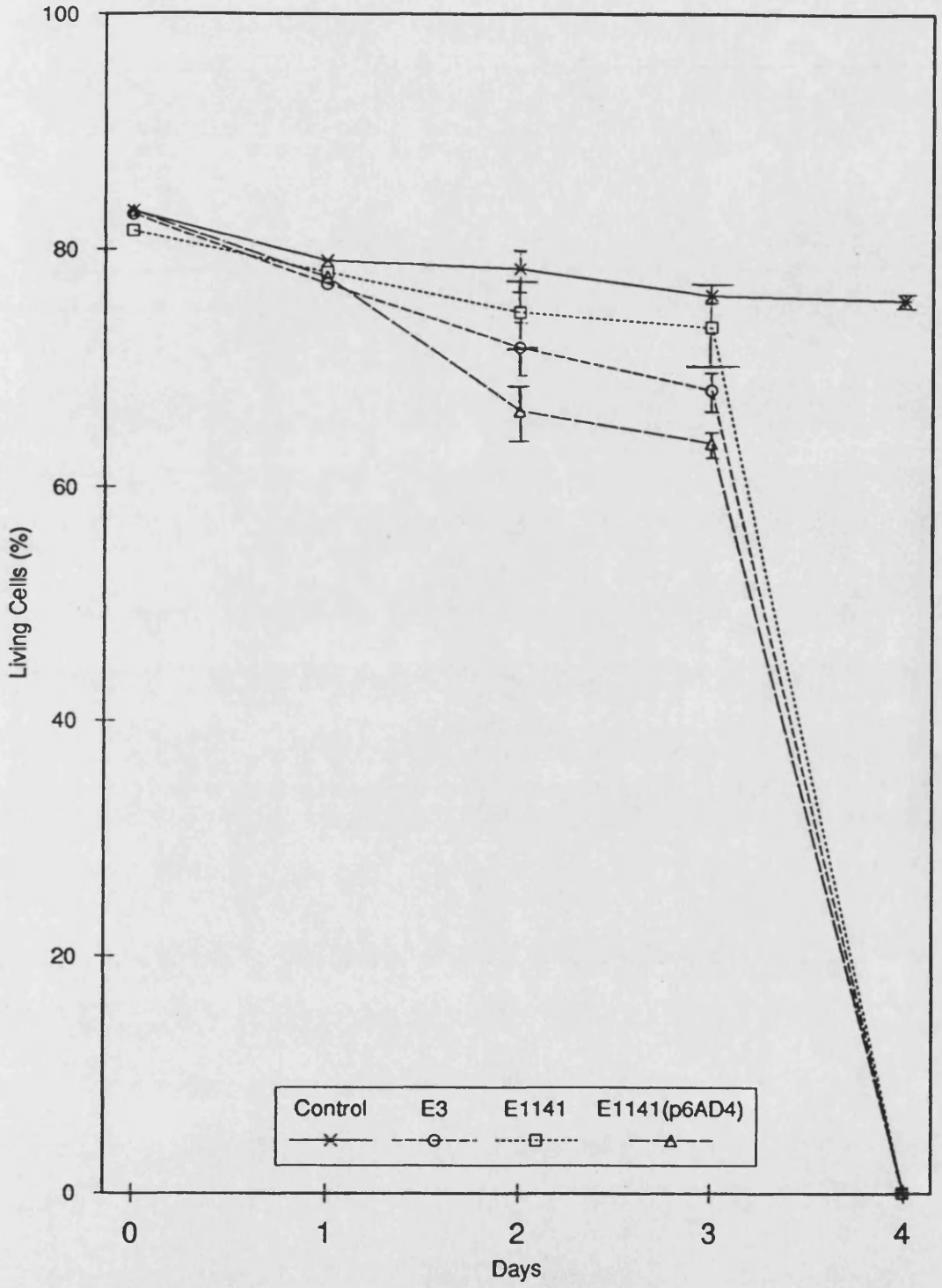


Figure 4.14: Bioassay of cell killing ability of *X. campestris* pv. *vesicatoria* strains E3, E1141, and E1141(p6AD4) on cells of tomato cv. Rootstock from stationary phase

10 ml of *Xcv* strains E3, E1141 and E1141(p6AD4) inocula (10^7 cfu ml^{-1}) were added to 5% pcv suspension cultures of tomato cv. Rootstock cells initiated from 18 day-old suspension cultures and incubated in an orbital shaker incubator 120 rpm, 16 hours photoperiod and light intensity was 150 $\mu E\ m^{-2}\ s^{-1}$. Means of 3 replicates are shown with the standard error bars.

Table 4.10: Number of *Xcv* strains E3, E1141 and E1141(p6AD4) survived in tomato cells (stationary phase) killing ability bioassay

Days	E3		E1141		$E1141_{(p6AD4)}$	
	With Tomato Cells	Control	With Tomato Cells	Control	With Tomato Cells	Control
0	1.4×10^7	1.3×10^7	1.1×10^7	1.2×10^7	1.1×10^7	1.3×10^7
1	1.2×10^7	1.0×10^7	1.1×10^7	1.0×10^7	1.4×10^7	1.3×10^7
2	2.2×10^8	1.0×10^6	1.3×10^8	1.0×10^6	1.0×10^8	2.3×10^6
3	2.6×10^8	3.4×10^5	2.7×10^8	6.3×10^5	1.0×10^8	2.1×10^5
4	2.2×10^8	1.1×10^5	1.9×10^8	1.0×10^5	1.0×10^8	1.7×10^5

The inoculum concentration of *Xcv* strains in 1.0 mM MES (pH 5.8) was 10^7 cfu ml^{-1} at the start of the experiment. The cultures were incubated in an orbital shaker 120 rpm at 25 ± 1 ^{0}C, 16 h photoperiod and light intensity of 150 μE m^{-2} s^{-1}. Samples were taken after two hours on day 0.

4.2.8 Production of Low Molecular Weight Toxin in Co-culture Medium of *Xcv* and Tomato Cells

To test the possibility that host cell killing was due to a low molecular weight toxin(s) the killing ability of E3 in direct contact with tomato cells was compared with E3 kept separately in sterilised dialysis tubing (12000-14000 daltons mass cut off). The dialysis tubing was cut into pieces of 10-20 cm, boiled for 10 minutes in a large volume of 2% sodium biocarbonate and 1 mM EDTA and rinsed thoroughly in distilled water. The tubing was then autoclaved for 10 minutes at 1.87 bar and 120 °C in a loosely capped jar filled with water, allowed to cool and stored at 5 °C. The tubing was kept submersed and washed thoroughly with distilled water. E3 inoculum was co-cultured with the suspension of tomato cells in direct contact and the same quantity of inoculum was transferred into the tubing and placed in the suspension of host cells. Assessment of the survival of tomato cells in both treatments and in distilled water showed that when E3 was kept in direct contact with tomato cells, the survival of the latter was reduced significantly ($p < 0.001$, Appendix 16). The effect of each treatment on the host cells was also significantly different with time ($p < 0.001$, Appendix 16) and this was clearly expressed 6 days after co-culture (Figure 4.15). The effect of E3 inoculum in tubing on the survival of tomato cells and distilled water followed the same pattern although the cell killing was higher in the former (Figure 4.15). This study suggests that either low molecular weight dialysable bacterial toxins were not responsible for all killing or that direct contact between host and pathogen was necessary to cause host cell killing. It is possible that the killing ability could be a result of high molecular weight molecules e.g. extracellular enzymes or polysacchrides.

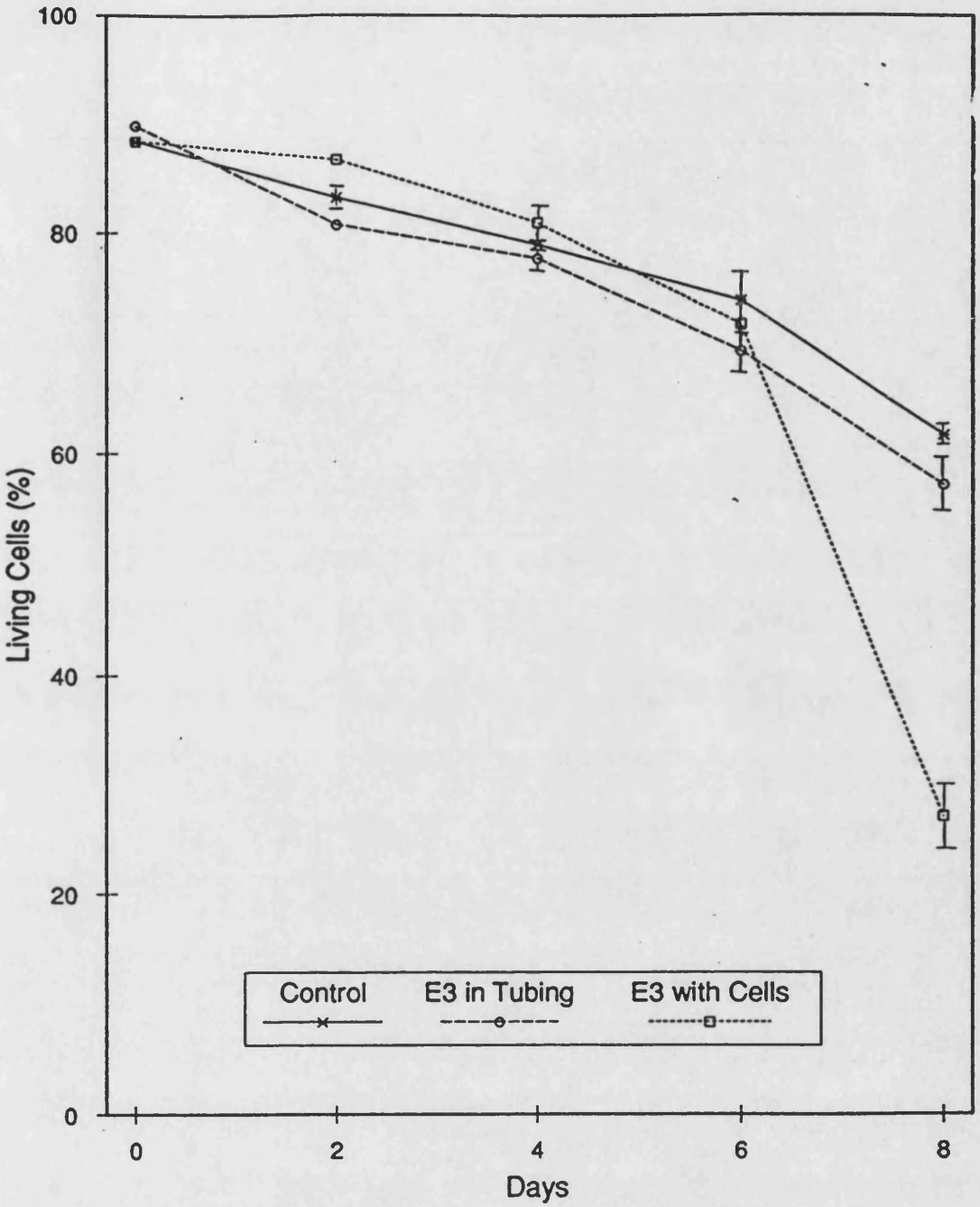


Figure 4.15: Survival of tomato cv. Rootstock cells (exponential phase) with and without physical contact between the cells and *X. campestris* pv. *vesicatoria* wild type E3

10 ml of *Xcv* strains E3 inoculum (10^7 cfu ml^{-1}) was added to 5% pcv suspension cultures of tomato cv. Rootstock cells initiated from 10 days-old suspension cultures and incubated in an orbital shaker incubator 120 rpm, 16 hours photoperiod and light intensity was 150 µE m^{-2} s^{-1}. Means of 3 replicates are shown with the standard error bars.

4.2.9 Regeneration of Tomato Cell Suspension Cultures

Tomato cells in the exponential phase of suspension culture showed resistance when co-cultured with pathogenic strains of *Xcv*. Regeneration of cells from this phase was necessary for testing the resistance at plant level. Three experiments were conducted with factorial combinations of cytokinins and auxins:

BAP (0, 0.1, 1.0, 10, and 100 μM) with NAA (0, 0.1, and 1.0 μM) on B5 medium with 15 replicates per treatment.

BAP (0, 0.10, 1.0, 10, and 100 μM) with IAA (0, 0.10, 1.0, and 10 μM) on MS medium with 10 replicates per treatment.

Zeatin (0, 0.1, 1.0, 10, and 100 μM) with IAA (0, 0.1, 1.0, and 10 μ M) on B5 medium with 8 replicates per treatment.

No organogenesis was observed in any of the three experiments after eight weeks, but a large amount of callus was produced in all treatments except the ones cultured on 100 μM of BAP or zeatin. On higher concentrations of cytokinins the callus showed brown colour one month after culture. Rootstock callus was maintained on 4.5 μM 2,4-D for long time. The difficulty of regenerating tomato callus produced by 2,4-D was discussed in Section 3.2.

4.3 Screening of Tomato Somaclones against *Xcv* Strain E3

Tomato somaclones (Sc_1) from cv. Rootstock and somaclones progenies (Sc_2) from cv. Moneymaker were screened against *Xcv* wild type E3. Sc_1 and Sc_2 plants were infiltrated with 10^8 cfu ml^{-1} of E3 after 3 weeks of transplanting and assessed for the development of leaf spot symptoms 2 and 8 days after infiltration.

4.3.1 Screening of Rootstock Somaclones (Sc_1) against *Xcv* Strain E3

Plants were regenerated from hypocotyl explants of tomato cv Rootstock. Three leaves on each of the 3 replicate plants were infiltrated with *Xcv* strain E3 (10^8 cfu ml^{-1}). The assessment of leaf spot symptoms was performed according to a 0-4, scale where 0 represents no disease symptoms and 4 represents severe disease symptoms. Water-soaking symptoms showed 2 days after infiltration on the leaves. The colour of infiltrated areas became brown at the middle and started to dry 3 days after infiltration. Chlorosis occured outside infiltrated areas 6 days after infiltration and expanded into uninfiltrated areas. The originally infiltrated areas were completely dry by this time. No difference between Rootstock somaclones was observed in their interaction with the E3, although there were genetic and morphological variations between these lines (Section 3.1.10.2).

4.3.2 Screening of Moneymaker Somaclones Progenies (Sc_2) against *Xcv* Strain E3

Tomato cv Moneymaker somaclones were selfed and seeds were collected. Five replicate seedlings grown from the seeds were infiltrated with 10^8 cfu ml^{-1} of E3 4 weeks after transplanting and incubated as described in Section (2.5.4). The assessment of leaf spot symptoms was performed according to a 0-4 scale described in Section (4.3.1). All Sc_2 seedlings were susceptible but two from the progenies of the tomato somaclone 21 showed an HR 2 days after infiltration (Table 4.11). Infiltrated areas collapsed and dried out (Plates 4.7a and b) 2 days after infiltration and no symptoms were observed outside infiltrated areas. Infiltrated leaves of the susceptible Sc_2 plants showed water soaking symptoms 2 days after infiltration and gradually dried out 4-6 day after infiltration. Extended chlorosis was shown outside the infiltrated areas 8 days after infiltration. Plants that showed HR were cloned for further investigations (cell leakage and population growth of *Xcv* strains), but cloned plants died 10 days after transplanting.

Table 4.11: Screening of tomato cv. Moneymaker somaclones progines (Sc_2) against *Xcv* E3

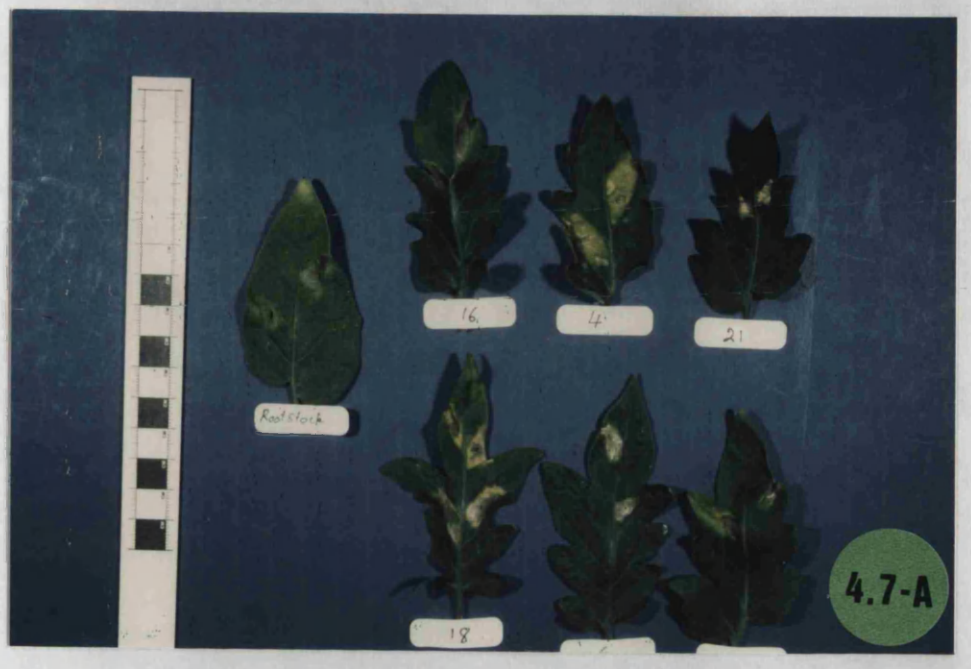
Sc_2 No.	Score		Sc_2 No.	Score		Sc_2 No.	Score	
	2 Days	8 days		2 days	8 Days		2 days	8 Days
1	0	4	17	0	4	31	0	4
2	0	4	18	0	4	32	0	4
4	0	4	20	0	4	33	0	4
5	0	4	21	2.5	4.5	34	0	4
6	0	4	22	0	4	35	0	4
8	0	4	23	0	4	36	0	4
9	0	4	24	0	4	37	0	4
10	0	4	25	0	4	38	0	4
11	0	4	26	0	4	39	0	4
12	0	4	27	0	4	40	0	4
14	0	4	28	0	4	41	0	4
15	0	4	29	0	4	42	0	4
16	0	4	30	0	4	M.Maker	0	4

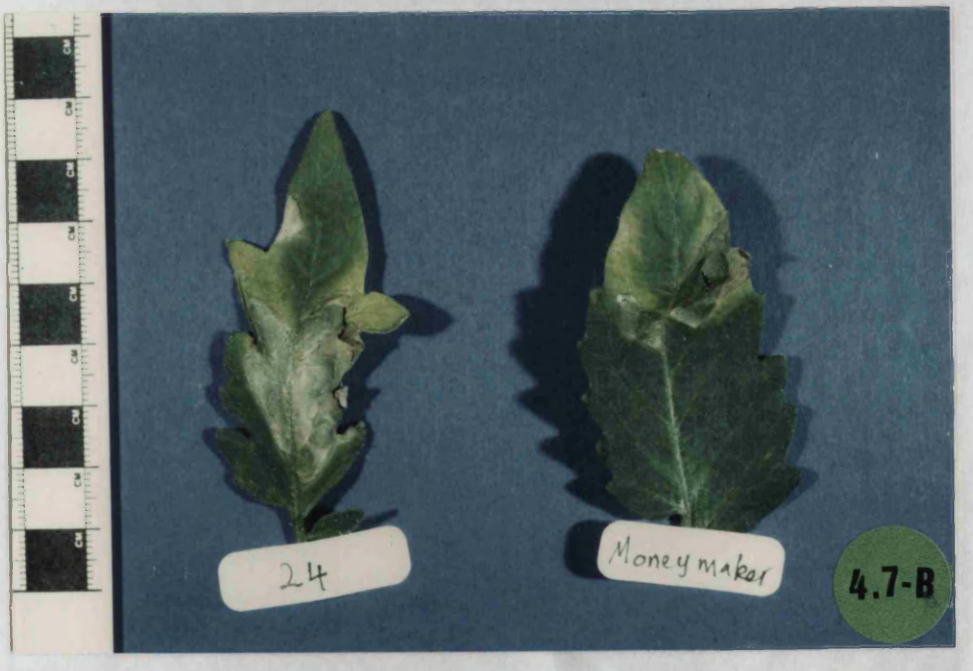
Seedlings the somaclones progenies regenerated from tomato cv. Moneymaker were inoculated with E3 (10^8 cfu ml^{-1}) and incubated at 25 ± 1 °C, 88-92% rh, 16 h photoperiod and light intensity of 180-250 μE m^{-2} s^{-1}. Plants were assessed for the development of disease symptoms 2 and 8 days after inoculation using the following scale:

0 = No symptoms; 1 = Lesions green, water-soaked, translucent, or just begining to turn brown, but with no chlorosis; 2 = Lesions brown and with small chlorotic halos; 3 = Lesions brown and conspicuous chlorosis around; 4 = Extensive chlorosis, affected portions may have turned brown and dried out, pronounced epinasty of leaflets and/or leaves.

Data are the means of 4 replicates for each treatment.

Plates 4.7a and b: Symptoms of leaf spot disease on leaves of tomato plants regenerated from cv. Moneymaker 8 days after infiltration with 10^8 Xcv E3


16
4
21
Rootstock
18
4.7-A


24
Money maker
4.7-B

that showed HR were cloned for further investigations (cell leakage and population growth of *Xcv* strains), but cloned plants died 10 days after transplanting. Thirty progenies of the same Sc_2 line (21) were screened against E3 following the same method described in this section to test if the resistance shown by this line against *Xcv* strain E3 was heritable. No HR was shown on leaves of any plant and all plants were found to be susceptible to the XCV. Chlorosis was observed outside the infiltrated area 8 days after infiltration and no difference between these plants in their interaction with E3.

4.4 Discussion

The non-pathogenic mutants E1004 and E1141 of *Xanthomonas campestris* pv. *vesicatoria* which were used in this study were derived from the wild type E3 by chemical mutagenesis and were identical to E3 in respect of growth rate *in vitro*, secretion of protease(s) and glucanase(s) and extracellular polysaccharides (Dymock and Clarkson personal communication). E3 is pathogenic on both tomato and pepper; and produces an HR in the resistant pepper cultivar ECW-10R, while the mutants E1004 and E1141 are non-pathogenic, and do not produce an HR in pepper ECW-10R. The pathogenicity and ability to produce an HR was restored in these mutants by the addition of the plasmid p6AD4 in *Xcv* strains E1004(p6AD4) and E1141(p6AD4). This plasmid also complements a Tn5 derived non-pathogenic mutant of *Xcv* and consists of 22 kb of *Xcv* DNA cloned into the broad host range vector pLAFR3 (Seal et al., 1990).

Upon infiltration into leaf tissue of the susceptible pepper ECW, the pathogenic strains of *Xanthomonas campestris* pv. *vesicatoria* E3 and E1141(p6AD4) undergo logarithmic growth until leaf spot symptoms developed. The population of these *Xcv* strains in tissues infiltrated with high inoculum concentration started to decrease when the host tissue dried out. Population growth

and cell leakage caused by E3 (wild type) were higher compared to the complemented mutant E1141(p6AD4), but both strains were higher in these aspects than the non-pathogenic mutant E1141. Reduction in population growth of the non-pathogenic *Xcv* mutant M461 was also reported by Seal et al. (1990) on both tomato and pepper ECW. The pathogenicity of M461 was restored by p6AD4, but to a lower level in comparison with the wild type 2595.

Results from an *in vitro* bacterial growth in NYGB and modified Watanabe broth suggested that the difference in population growth *in planta* was unlikely to be due to differences in level of nutrients, but to the ability of the strain to cause damage to the host cells and gain access to nutrients.

The HR is a rapid response of infected plant tissue in an incompatible interaction with a pathogen. In an HR, inoculated tissue collapses rapidly, a rapid increase in cell leakage and multiplication of the pathogen is restricted in the tissue (Stall and Cook, 1966; Scharen, 1959; Cook, 1973; Klement and Goodman, 1967; Stall et al., 1974; Kim and Hartmann, 1985). The pathogenic *Xcv* strains E3 and E1141(p6AD4) also induced an HR on ECW-10R 24 h after infiltration with 10^8 cfu ml^{-1}, but E1141 did not. Scharen (1959) reported that a temporary reduction of *Xanthomonas phaseoli* population was caused by host reaction in susceptible and resistant hosts, and further multiplication was restricted in the resistant host. Seal et al. (1990) suggested that the inability of M641, a non-pathogenic mutant, to cause an HR in pepper was due to its inability to grow to the level sufficient to cause an HR, since high inoculum (10^9 cfu ml^{-1}) of M461 induced an HR. The inability to restore the pathogenicity of mutated *Xcv* strain E1141(p6AD4) to the complete wild type level with p6AD4 may be due to the loss of the plasmid under the non-selective growth conditions *in planta.* It is also possible that there are additional pathogenicity gene mutations apart from that complemented by p6AD4. The results of Bonas et al. (1991) also correspond with the findings in this study. They reported that non-pathogenic

mutants failed to grow to the wild type level and behave saprophytically *in planta* without development of disease symptoms on leaf tissue of pepper ECW infiltrated with high densities (10^8-10^{10} cfu ml^{-1}) of these mutants. The molecular and biochemical function of avirulent genes and the pathway of an HR induction is awaiting further research.

The rate of population growth was higher when low concentrations of inocula were infiltrated in the leaf tissue of ECW. From the different inoculum concentrations, it appeared that the length of time needed to reach the maximum population and symptom development varied with the initial number of bacteria infiltrated in the leaf tissue. The time required to reach maximum cell numbers with low inoculum concentrations of pathogenic strains was 8-9 days compared to 2-3 days for high inoculum concentrations. A simultaneous increase in cell leakage was observed on each tissue inoculated with pathogenic strains. A correlation between the low population of the non-pathogenic *Xcv* strain E1141 and low cell leakage was clearly established. Inoculation methods other than leaf infiltration proved to be less effective in introducing the number of bacteria sufficient to develop disease symptoms.

The interaction of the three strains of *Xcv*; E3, E1141 and E1141(p6AD4) with susceptible pepper ECW and resistant pepper ECW-10R was compared by infiltrating the leaves of these lines with 10^8 cfu ml^{-1} of each strain. An HR was induced on ECW-10R leaves after 24 h of infiltration with pathogenic strains E3 and E1141(p6AD4), but no disease symptoms developed on the leaves of ECW-10R infiltrated with E1141. However, no symptom developed on leaves of the susceptible pepper cv. ECW infiltrated with any of the three *Xcv* strains within the 24 h period. An HR on leaf spot resistant pepper infiltrated with an avirulent strain of *Xcv* has been reported by many authors (Stall and Cook, 1966; Stall et al., 1974; Cook, 1973; Cook and Guevara, 1984). Klement and Goodman (1967) reported an HR on tobacco infiltrated with *Pseudomonas syringae* and

Erwinia amylovora (both are non-pathogenic on tobacco). In these reports low bacterial populations, rapid increase in cell leakage and localized necrotic regions were the main features of an HR. Because of the symptoms of an HR on ECW-10R induced by the pathogenic strains E3 and E1141(p6AD4) in this study, it is believed that the same mechanisms of host-pathogen interaction were operating. Distinct forms of HR induced on resistant and susceptiple genotypes of pepper inoculated with naturally occuring pathotypes of *Xcv* (pepper and tomato strains) was also reported by Cook (1973). He found that tissues killed by the tomato isolate 71-14 were darker than tissues killed by the pepper strain. In addition the patterns of electrolyte leakage from both pepper cultivars inoculated with the tomato isolates were intermediate to those established for the pepper strain, race 2, on similar pepper genotypes.

When low and high inoculum concentrations of E3, E1141(p6AD4) and E1141 were infiltrated into leaf tissue of susceptible tomato cv. Rootstock, the general patterns of population growth and cell damage were similar to those in the susceptible pepper cultivar ECW. In contrast to cv. ECW, no leaf shedding occured with the highest inoculum concentration. Maximum bacterial numbers were 100 fold lower than that on cv. ECW. The highest growth rate was observed when a low inoculum concentration (10^6 cfu ml^{-1}) of E3 was used, but the population of E1141(p6AD4) infiltrated at the same concentration increased for the first 2 days after infiltration and then started to decrease. This could be due to the loss of p6AD4 plasmid and/or an inability to grow to the same level as E3. However, cell leakage caused by low inoculum concentrations of these pathogenic strains increased steadily in a similar pattern irrespective of the population growth. Population growth and cell leakage were low for the non-pathogenic strain E1141 and no symptoms developed on tissue infiltrated with this strain at both inoculum concentrations. Wounding or epidermal hair breakage proved to be necessary for successful inoculation of tomato, as was reported by

tomato cv. Hawaii 7998, while the population of the non-pathogenic strain E1141 increased steadily at low and high inoculum concentrations. The reduction in population growth was accompanied by an increase in cell leakage, suggesting an HR. However, cell leakage remained unchanged in tissues of Hawaii 7998 infiltrated with a high inoculum concentration of the *Xcv* strain E1141 (non-pathogenic) and no disease symptoms were induced. In contrast, Jones and Scott (1986) did not observe differences in bacterial populations between resistant and susceptible tomato 7 days after infiltration with 10^8 cfu ml^{-1}, but infiltration with a low concentration of bacteria (10^5 cfu ml^{-1}) showed dramatic differences between these lines. The results of bacterial population growth and cell leakage on cv. Hawaii 7998 suggest that the pathogenic strains of *Xcv* were recognized by this cultivar and the restriction of population growth resulted from the incompatible host-pathogen interaction. However, the non-pathogenic strain E1141 was not recognized by cv. Hawaii 7998 and was able to grow in a saprophytic manner. Low inoculum concentrations of the pathogenic bacteria did not grow to the threshold that was sufficient to trigger an HR.

The results of cell leakage on cv. Hawaii 7998 were generally in agreement with Jones and Scott (1986) who reported a gradual increase in electrolyte leakage of Hawaii 7998 24 h after infiltration with pathogenic *Xcv* (10^8 cfu ml^{-1}). The difference in HR patterns could be a result of the different *Xcv* strains, since Hawaii 7998 was reported to be susceptible to some *Xcv* strains (Ward and O'Garro, 1992). The resistance of cv. Hawaii 7998 to *Xcv* is reported to be controlled by an additive gene effect (Scott and Jones, 1989) and consequently the HR may not be typical. Minsavage et al. (1990) reported different patterns of cell leakage on pepper as well as variation in the time of an HR appearance, and the intensity of browning upon the interaction between avirulent genes of *Xcv* strains and resistance genes of pepper.

In contrast to the limited population growth in resistant tomato cv. Hawaii

and the intensity of browning upon the interaction between avirulent genes of *Xcv* strains and resistance genes of pepper.

In contrast to the limited population growth in resistant tomato cv. Hawaii 7998, Parry and Callow (1986) found little difference in the multiplication and spread of *Xanthomonas campestris* pv. *oryzae* in rice during either a compatible or incompatible interaction, although the lesion lengths were substantially different. They concluded that the expression of resistance in rice was not characterized by a lower bacterial population but was the result of reduced symptom expression by the host. However, Barton-Willis et al. (1989) reported that the numbers of *Xanthomonas campestris* pv. *oryzae* increase equally in compatible and incompatible interactions until a certain level (10^7-10^8 cfu per leaf) after which the growth rate of incompatible interaction slowed down.

The gradual increase in cell leakage and relatively longer time to induce an HR are in agreement with Minsavage et al. (1990) and Essenberge et al. (1979). In contrast, Essenberge et al. (1979) found that the final population density in immune cotton leaves inoculated with *Xanthomonas malvacearum* was independent of inoculum density. Schulte and Bonas (1992) reported that, the *hrp* gene cluster from *Xcv* determines the pathogenicity on the host plants pepper and tomato and the induction of an HR on resistant host and nonhost plants. This is believed to be the case in the *Xcv* mutants used in this study. The expression of the *hrp* genes was found to be high when the bacteria were grown *in planta*, but this expression was not detectable when the bacteria were grown in complex or minimum medium (Schulte and Bonas, 1992).

The interaction between *Xcv* and tomato was also studied *in vitro*. The survival in cv. Rootstock cells and the length of time needed for a substantial reduction of the number of living cells varied with the age of tomato cells. Cells of tomato cv. Rootstock from the exponential phase of suspension culture were found to be more resistant to *Xcv* than cells taken from lag and stationary phases.

cells from lag and exponential phases of suspension growth were reduced substantially 6 and 8 days respectively, when co-cultured with pathogenic strains of *Xcv*. Plants regenerated from tomato cells which showed more resistance to the *Xcv* strains could have shown an increase in the level of resistance to *Xcv*. The regeneration of these cells has been difficult and more work is needed to achieve this goal.

A successful *in vitro* approach for the selection of peach plants resistant to *Xanthomonas campestris* pv. *pruni* was developed by Hammerschlag (1984; 1988). Hammerschlag (1984) reported a toxic metabolite(s) in the culture filtrate of *Xanthomonas campestris* pv. *pruni*. This metabolite(s) proved to play a role in bacterial spot of peach and was active at the cellular level. The non-specific toxin produced by *Xanthomonas campestris* pv. *pruni* was used in a selection programme and resistant peach plants were regenerated from the filtrate resistant-calli (Hammerschlag, 1988).

No toxins have been characterised for *Xcv* and the results of this study suggest that low molecular weight molecules were not involved in the killing of tomato cells *in vitro*. In contrast, Hammerschlag (1984; 1988) reported that a low molecular weight fraction of the culture filtrate of *X. campestris pv. pruni* was an effective screening agent in a cell selection programme for resistance against the pathogen. Schulte and Bonas (1992) reported that filtrates of pepper, tomato, and tobacco cell suspension cultures contain a molecule(s) that induces *hrp* gene expression and the inducing molecule was found to be heat stable, hydrophilic and have low molecular weight. Therefore induction of pathogenicity of *Xcv* retained in dialysis tubing, as described in this study, would be expected. However host cell killing did not occur, suggesting that high molecular weight factor(s) are required for pathogenicity. Culture filtrates should be tested in future experiments and may be useful in the selection of disease resistant cell lines. Regeneration of tomato plants resistant to bacterial wilt from calli resistant to the

are required for pathogenicity. Culture filtrates should be tested in future experiments and may be useful in the selection of disease resistant cell lines. Regeneration of tomato plants resistant to bacterial wilt from calli resistant to the culture filtrate of *Pseudomonas solanacearum* has been reported (Toyoda et al., 1989).

Although tomato plants were not regenerated from callus maintained on 2,4-D for long periods, it was possible to regenerate from tomato (cvs Money-maker and Rootstock) explants after a brief period of callus growth. Progenies of Moneymaker somaclones showed phenotypic variation in their interaction with *Xcv* strain E3, but further analysis of these differences were hindered by the death of these plants in the greenhouse. These results suggested the possibility of regenerating plants with improved characters through tissue culture, but the use of somaclonal variation requires the screening of a large number of plants to find the required change. Only a limited number of somaclones were screened against *Xcv* strain E3 from each tomato cultivar. Wright and Lacy (1988) reported an increase in the level of disease resistance against 3 fungal and one bacterial diseases in Celery plants regenerated from cell suspension cultures. In these somaclones resistance to a given pathogen appeared to arise independently from resistance to other pathogens, since plants with high resistance to two or more than two pathogens were rarely found.

The results of this study suggested a correlation between the interaction of *Xcv* strains *in planta* and their interaction with tomato cells *in vitro*. The pathogenic *Xcv* strains infiltrated in leaf tissues of susceptible pepper and tomato caused cell leakage. These pathogenic strains also caused the death of tomato cv. Rootstock cells in suspension cultures. The bacterial numbers increased in both interactions. The potential use of somaclonal variation for disease resistance was also demonstrated on plants regenerated from explants of tomato cv. Moneymaker

which showed variation in their interaction with *Xcv* strain E3. Cells of tomato cv. Rootstock from different growth phases of suspension cultures also showed variation in their interaction with the pathogenic *Xcv* strains.

Chapter (5)

Tomato Powdery Mildew caused by *E. cichoracearum*

5.1 Results

5.1.1 Effect of Temperature and Relative Humidity on Conidia Germination and Germ Tube Elongation

Geographical distribution of the 2 different species of tomato powdery mildew presumably results from the effects of climate (relative humidity (rh), temperature and light), but there is no evidence to support this hypothesis. The effect of temperature and humidity on powdery mildew conidia germination were therefore investigated; this information would also provide the optimum conditions in which this obligate parasite could be kept and for the screening of tomato regenerants against the parasite. Unfortunately only the *Erysiphe sp.* could be studied because of non-availability of *L. taurica.*

The powdery mildew inoculum was maintained on tomato cv. Moneymaker plants in a Fisons growth chamber (Section 2.6.1). The rh control and germination of powdery mildew conidia on glass were done as in Section 2.6.2. The effects of 8 humidity levels (5, 20, 40, 60, 80, 90 and 100%) were tested in a dark incubator at 10, 15, 20, 25, 30, 35 and 40 °C. A conidium was considered germinated when its germ tube length was longer than its breadth. For the determination of germination, 300 conidia from each of three replicate chambers were counted from three low power fields under the light microscope. In tests at 100% rh., fields relatively free from condensation were selected. Twenty five germ tube lengths were measured per replicate. The complete randomised design was used for the data analysis and the means were tested according to Duncan Multiple Range Test. Vapour pressure deficit (VPD) was calculated and is shown

on Figures 5.1-5.5. VPD was calculated according to the following formula Vpd = (1-rh)E where rh = relative humidity and E = vapour pressure at saturation at a given temperature.

The germination of *E. cichoracearum* conidia was affected by both temperature and rh levels. No conidial germination was observed at 10 and 40 °C irrespective of the humidity. Generally, conidial germination increased with the increase in rh level. When germination was tested at different humidities at 15 °C, no germination was observed with ≤ 40% rh, but it increased significantly ($p < 0.001$) with rh levels above 60% (Figure 5.1a) e.g. the germination was 1.7% at 60% compared to 14.3% when the humidity was increased to 100%. The germ tube lengths at 15 °C increased significantly ($p < 0.01$) with increasing rh and the longest germ tubes (24.5 µm were observed at 90% rh followed by 80, 100 and 60% (Figure 5.1b). Conidia germinated at all humidity levels tested at 20 °C and the germination increased significantly ($p < 0.001$) with the increase in rh from 0.7% at 5% rh to 96% at 100% rh (Figure 5.2a). The germ tube lengths also increased significantly ($p < 0.001$) with rh from 4 µm at 5% rh to 63.1 µm at 100% (Figure 5.2b). There were marked differences in conidial germination and germ tube lengths at 100 and 40% rh; germination was higher (96.0%) and germ tubes longer (63.1 µm) compared to 15.7% germination and 26.0 µm germ tube lengths respectively (Plates 5.1 and 5.2). The same pattern of germination increase with the humidity level was repeated at 25 °C, but the germination was lower than 20 °C. The germination increased significantly ($p < 0.001$) from 1.7 to 90.3% at 5% and 100% rh respectively (Figure 5.3a). However, germ tubes were longer at 25 °C compared to those at 20 °C when incubated at ≥ 90% rh. At 25 °C the germ tube lengths were 57.9, 65.2 and 70.0 µm at 90, 95 and 100% rh respectively compared to 47.5, 57.2 and 63.1 µm at these rh at 20 °C. In addition the germ tube lengths at low humidity (5%) at 25 °C was longer (17.0 µm) than that developed at 20 °C (4.0µm). Germ tube

Figure 5.1-5.5: Effect of temperature and humidity on the germination of *E. cichoracearum* conidia and germ tubes elongation

Conidia were collected on cover slips from diseased plants and germinated in humidity chambers containing solutions of NaOH which maintained the humidity at the required levels. The germination of 100 conidia and length of 25 germ tube were assessed per replicate after 24 h of incubation. Analysis of data was performed according to complete randomized design and Duncan Multiple range Test was used for mean separation. Data represent the means and standard errors of 3 replicates.

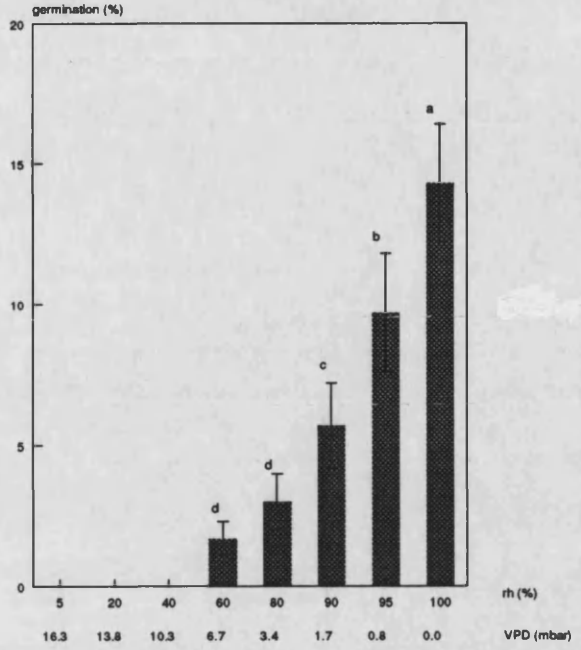


Figure 5.1a: Effect of humidity on germination of powdery mildew conidia at 15 °C

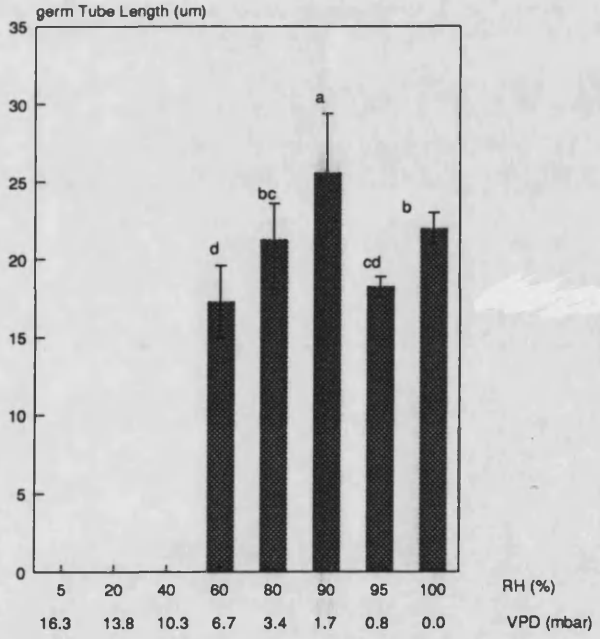


Figure 5.1b: Effect of humidity on the elongation of powdery mildew germ tubes at 15 °C

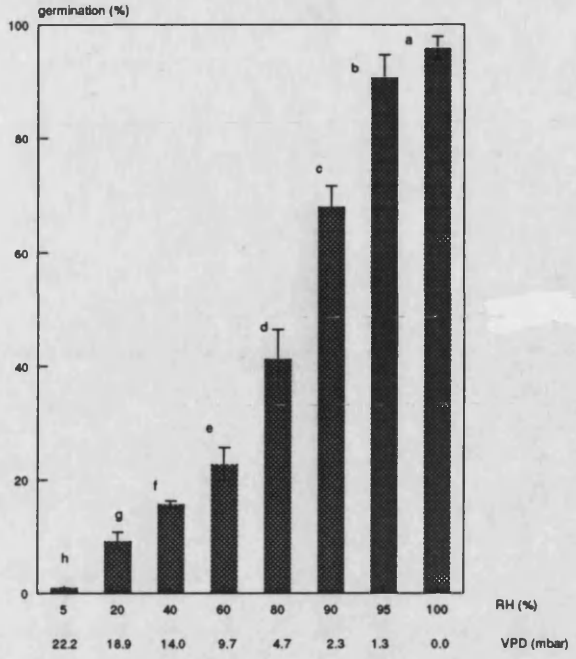


Figure 5.2a: Effect of humidity on germination of powdery mildew conidia at 20 °C

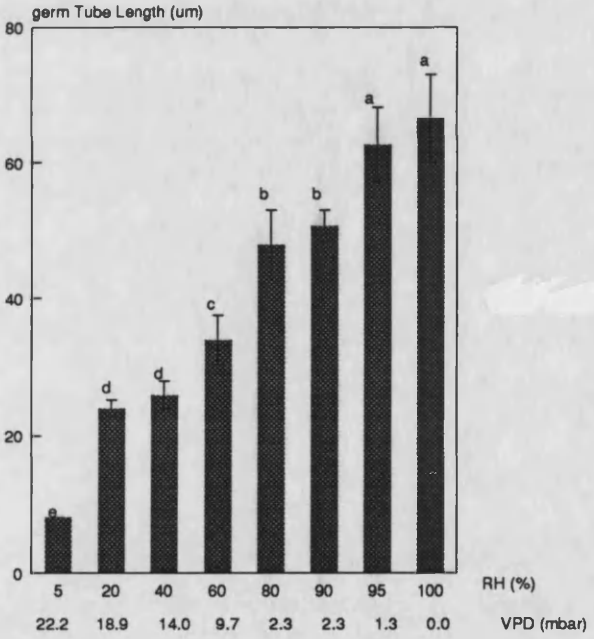


Figure 5.2b: Effect of humiditppby on the elongation of powdery mildew germ tubes at 20 °C

Plate 5.1: Germination of powdery mildew conidia on glass at 20 °C and 20% relative humidity 24 h after incubation

Most of conidia were shrivelled and few germinated

Plate 5.2: Germination of powdery mildew conidia on glass at 20 °C and 100% relative humidity 24 h after incubation

Powdery mildew conidia were turgid and germinated. A short germ-tube was generated at one end of each conidium. An appressorium was formed on the end of the germ tube of most germinated conidia. Occasionally secondary hyphae developed from the same end where the germ tube was generated.

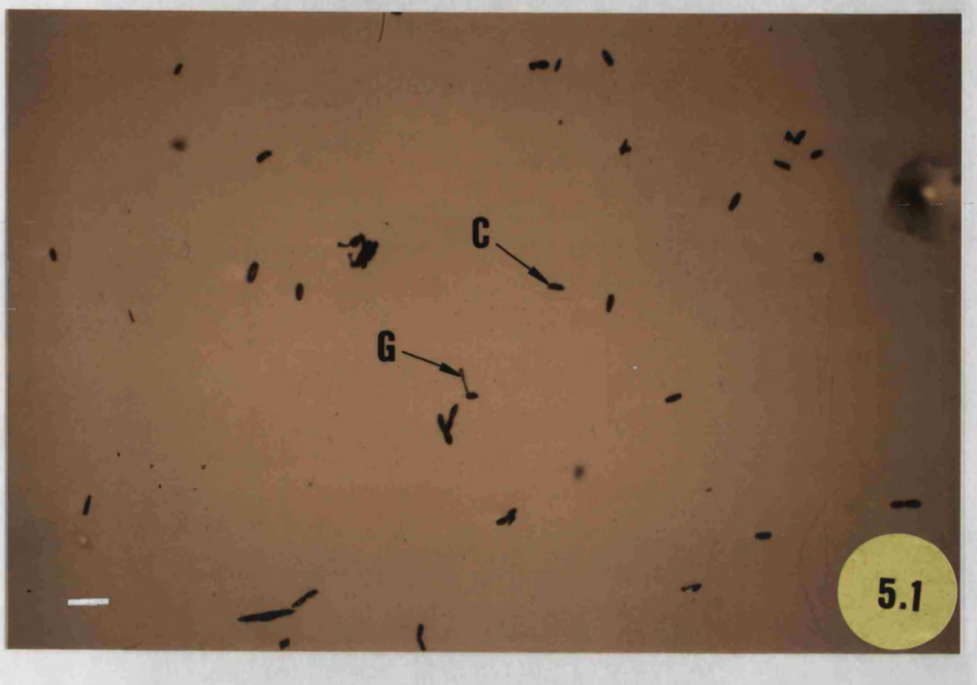
Bar = 100 μ

C = Conidium

G = Germ tube

AP = Appressorium

S = Secondary hypha


C
G
5.1


S
AP
C
G
5.2

lengths increased significantly ($p < 0.001$) with the increase in humidity level at 25 °C (Figure 5.3b). The germ tube lengths increased from 17.0 to 70.0 μm at 5% and 100% rh respectively.

At both 20 and 25 °C appressoria formed at the end of short germ tubes at only 100% rh along with long germ tubes that had no appressoria.

The germination of conidia at 30 °C also increased significantly ($p < 0.001$) with rh, but it remained lower than at 20 and 25 °C. It increased from 2.0 to 66.0% at 5 and 100% rh respectively (Figure 5.4a); This compares with germination at 100% rh at 25 °C of 90.3%. The germ tube lengths increased significantly ($p < 0.001$) with humidity level (Figure 5.4b) from 16 to 64.6 μm following increase of rh from 5 to 100% at 30 °C. Conidia incubated at 35 °C at 5% rh failed to germinate. However, the germination of conidia incubated ≥ 20% increased significantly ($p < 0.001$) from 4.7 to 36.3% at 20 and 100% rh respectively (Figure 5.5a). The germ tubes growth was similar at all humidity levels tested at 35 °C (Figure 5.5b).

Thus the optimum temperature for the germination of conidia and germ tubes elongation was 20 and 25 °C respectively and the optimum rh was 100%. The temperature limits at which conidia can germinate are ≥ 15 and ≤ 35 °C and the rh ≥ 5 and ≤ 100%.

5.1.2 Effect of Different Humidity Levels on Germ Tube Growth at 20 °C

The optimum temperature for conidial germination on glass was shown above to be 20 °C; but the germ tube lengths were significantly different at 40, 60 and 95% rh at this temperature. Therefore the growth of germ tubes was investigated at 20 °C and 40, 60 and 95% rh. Collection of inoculum, humidity control and incubation were performed as in Section 2.6.1. The germ tube lengths were measured for 100 conidia hourly for 9 hours and then after 24 hours with three replicates each time.

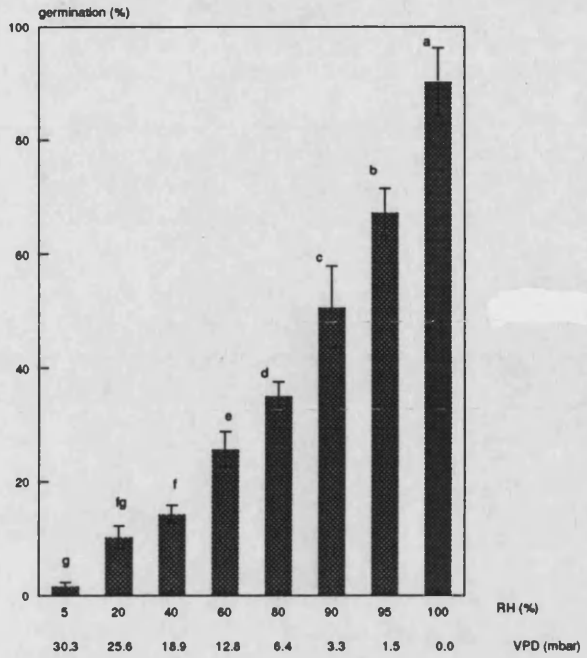


Figure 5.3a: Effect of humidity on germination of powdery mildew conidia at 25 °C

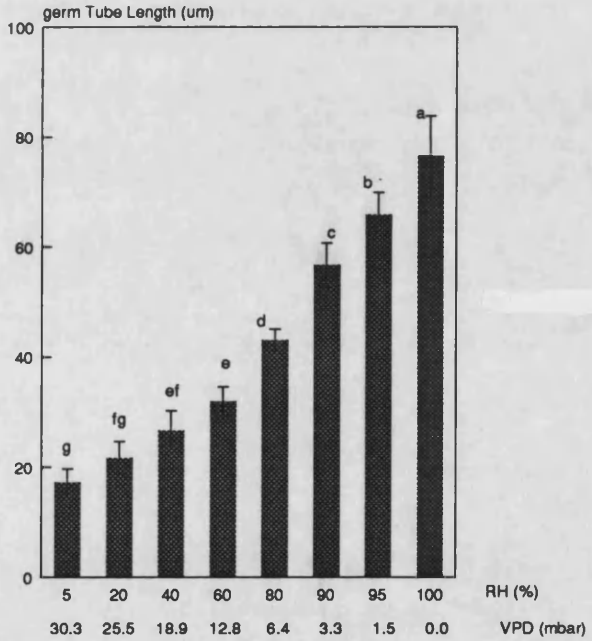


Figure 5.3b: Effect of humidity on the elongation of powdery mildew germ tubes at 25 °C

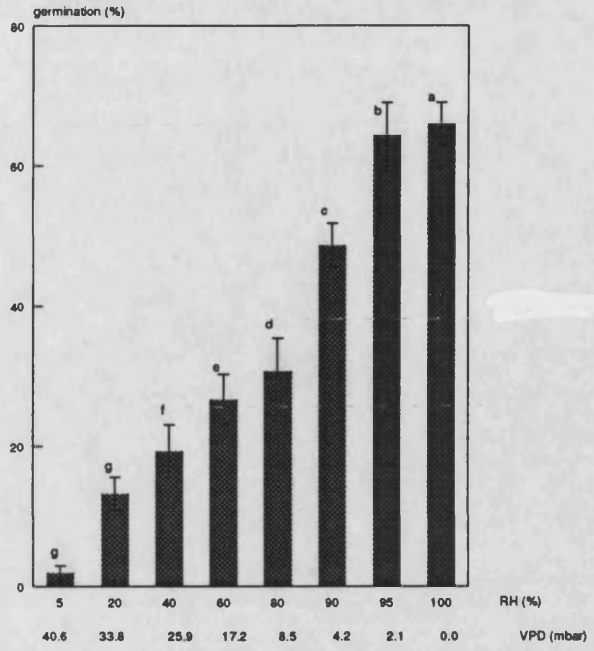


Figure 5.4a: Effect of humidity on germination of powdery mildew conidia at 30 °C

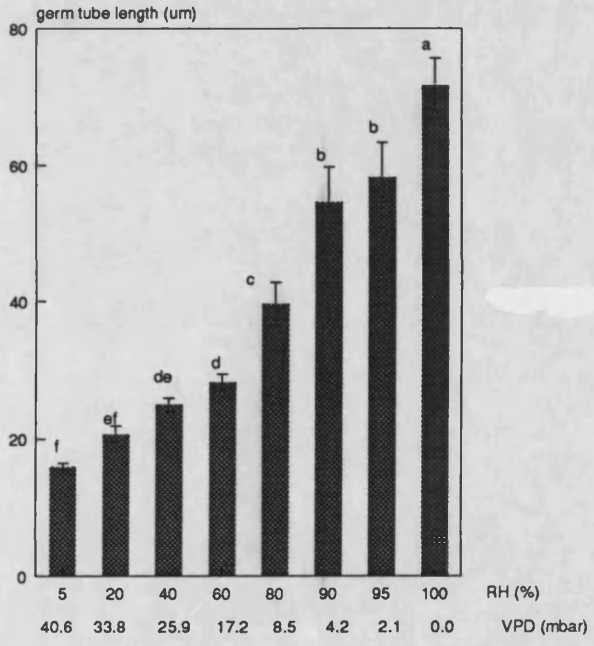


Figure 5.4b: Effect of humidity on the elongation of powdery mildew germ tubes at 30 °C

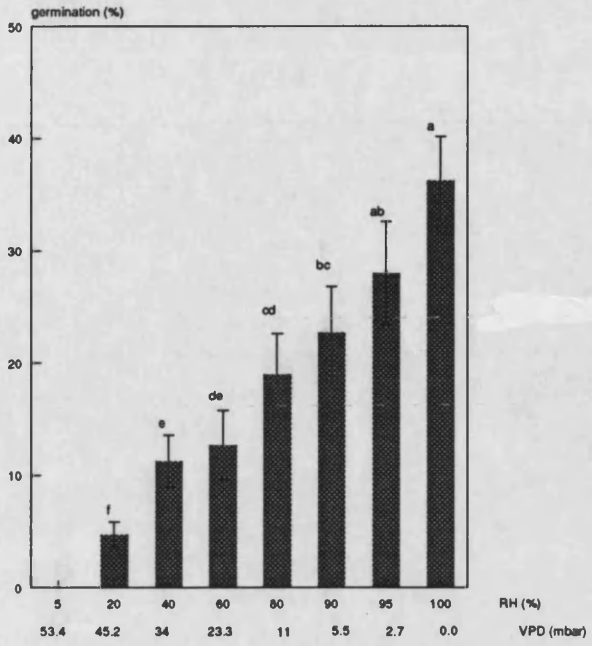


Figure 5.5a: Effect of humidity on germination of powdery mildew conidia at 35° C

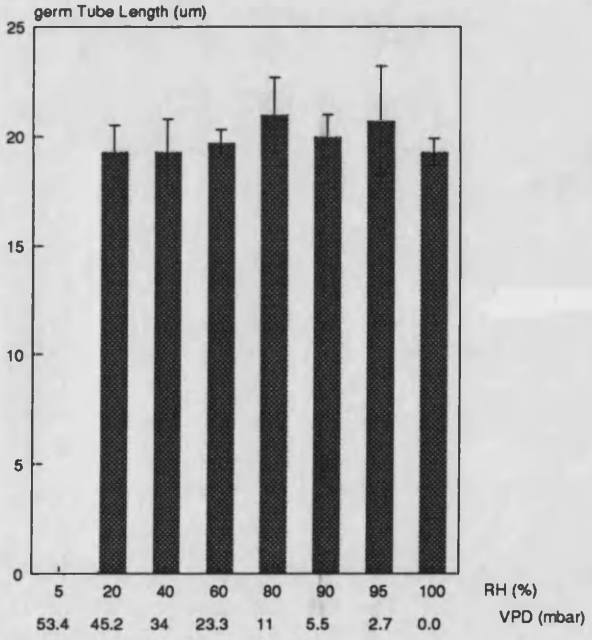


Figure 5.5b: Effect of humidity on the elongation of powdery mildew germ tubes at 35° C

Conidia germination started between 3 to 4 hours at 60 and 95% rh, but 4 to 5 hours were needed for initiation of germination of the conidia incubated at 40% rh Germ tubes grew to twice the lengths of conidia in 2 h after germination, at 60 and 95% rh. After 4 hours, germ tube lengths were 28.0, 30.4 and 47.2 μm at 40, 60 and 95% rh (Figure 5.6). At 40% rh germ tubes did not elongate further. Germ tubes grew to 28.8, 46.4 and 68.0 μ m at 40, 60 and 95% rh respectively after 24 hours. A few conidia (< 2%) of those incubated at 95% humidity had short germ tubes which gave rise to forked appressoria formed on glass slides.

5.1.3 Primary Infection of Powdery Mildew *E. cichoracearum* on Tomato cv. Moneymaker

This experiment was done to investigate the primary infection stages of powdery mildew on tomato leaves and to compare rate and extent of germination and appressorium formation with that *in vitro*. Six week old tomato seedlings were inoculated with powdery mildew conidia from infected plants (section 2.5.1). Four seedlings per replicate were incubated as in Section 2.6.1, and 4 leaf discs (4 mm diameter) were taken from each of the inoculated plants every two hours for microscopic observation of conidial germination and the development of fungal structures (Section 2.6.4).

On tomato leaves 9.1% of the conidia germinated after two hours of inoculation, and germination then increased with time to reach 66.1, 72.6, 86.7 and 89.1% after 8, 16, 24 and 30 hours respectively (Figure 5.7). Germ tubes emerged laterally near one end of the conidia. Formation of lobed adhesive bodies (appressoria) at the apices of germ tubes increased with time and 4.7% germinated conidia formed appressoria after two hours of incubation which increased to 36.0, 56.8, 74.7 and 83.6% after 8, 16, 24 and 30 hours respectively. The appressoria took about 16 hours to mature and attach to the leaf surface. After 18 hours

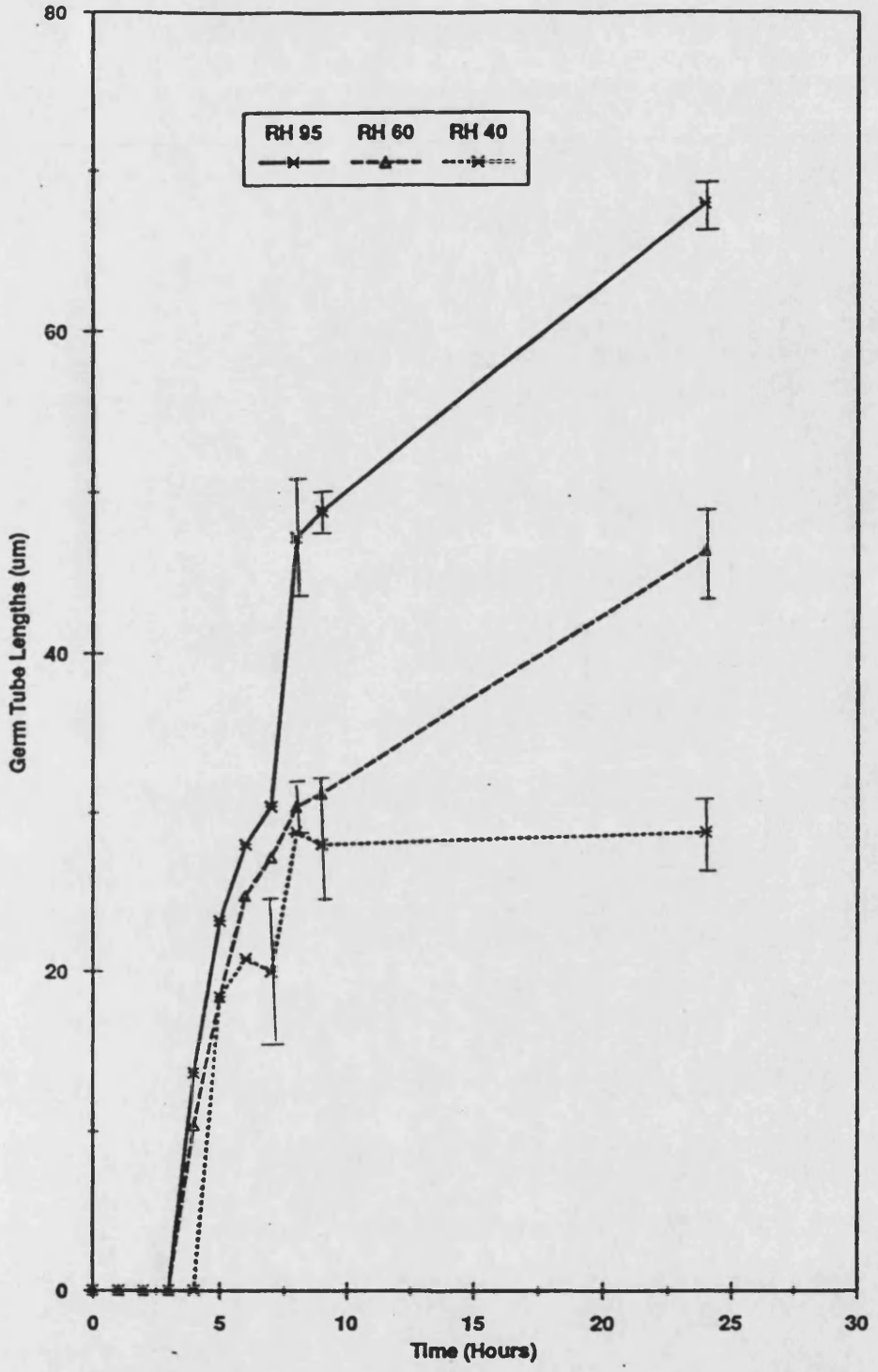


Figure 5.6: Elongation of powdery mildew germ tubes at 20° C and different humidity levels

The lengths of 100 germ tube was measured per replicate. Data are the means and standard errors of 3 replicates

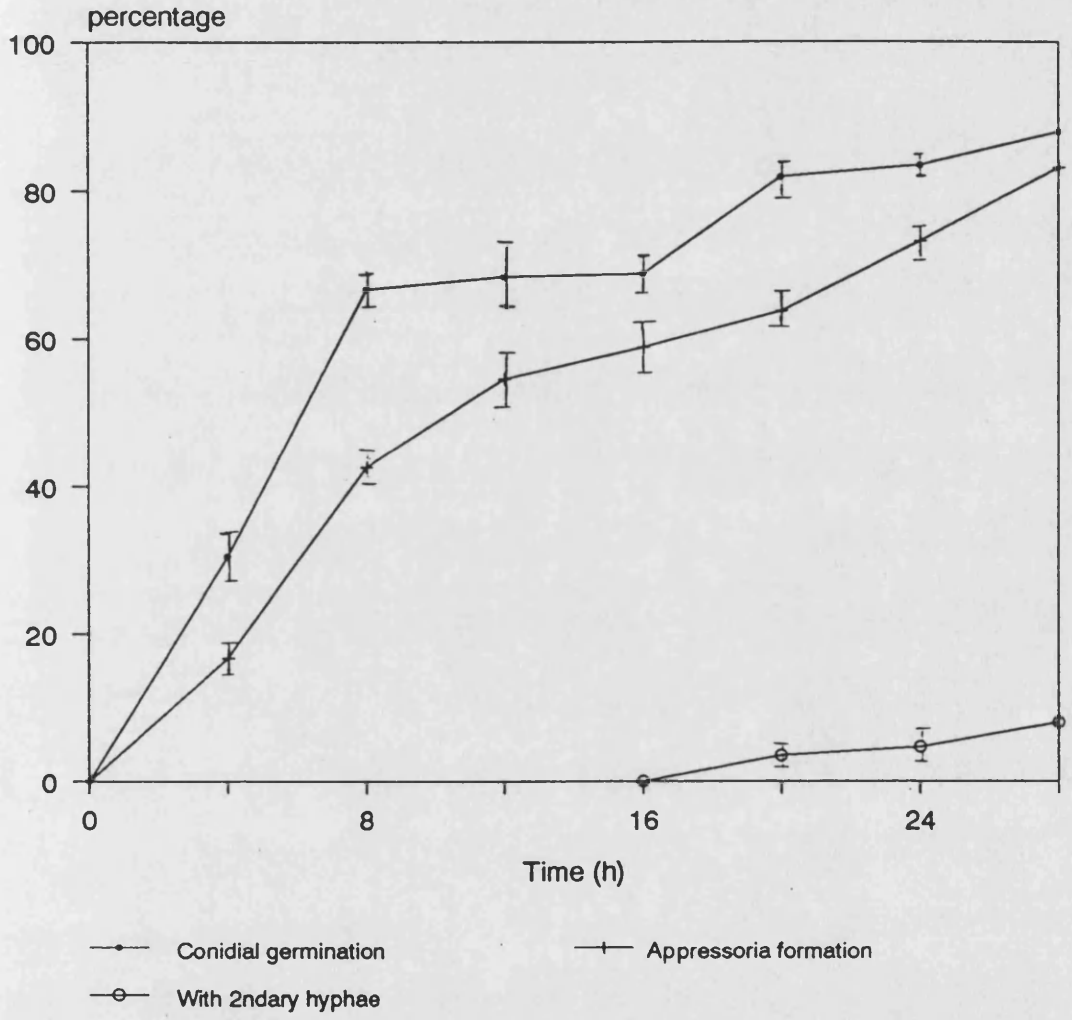


Figure 5.7: Primary infection of powdery mildew on tomato cv. Moneymaker

Plants were inoculated with conidia from diseased plants and incubated at 24 °C, 16 h day, 88-92% rh and light intensity 150 µE m-2 s-1. The primary infection of of powdery mildew was assessed on leaf discs from 3 replicates (200 conidia / replicate).

secondary hyphae were produced from 2.5% of the germinated conidia and these increased to 7.96% after 30 hours; one to two secondary hyphae were produced per conidium.

These hyphae usually emerged from appressoria, grew across the leaf surface and probably penetrated the leaf surface directly because no penetration through stomata was found.

5.1.4 Screening of Wild Genotypes of Tomato against Powdery Mildew

Seedlings of susceptible tomato cv. Moneymaker and two wild genotypes of tomato LA2747 and LA458 were screened against the pathogen. The wild lines were among five genotypes which have shown some resistance to powdery mildew according to initial screening (Rick, personal communication); other wild lines did not produce enough seedlings because of the low seed germination. Four seedlings from these wild lines and Moneymaker seedlings (4 weeks-old) were inoculated with powdery mildew conidia and incubated as described in Section 2.6.1, and the plants were observed after 10 days for disease symptoms. Number of powdery mildew colonies, disease incidence and dead leaves per plant were recorded in addition to disease assessment using 0-5 scale, where 0 represented no symptoms; 1 when 1-4% of the foliage surface was covered with mildew; 2: 5-10% covered; 3: 11-25% covered; 4: 26-50% covered and 5: represented more than 50% of the foliage surface was covered with mildew.

Disease symptoms developed on the wild lines LA2747 and LA458 as well as on Moneymaker seedlings after 10 days. Powdery mildew colonies developed per plant were significantly ($p < 0.05$) higher on Moneymaker (516.5 colonies) compared to LA2747 and LA458 which had only 68.8 and 50.3 colonies per plant respectively (Table 5.1). However, the wild lines were not different from each other. Disease incidence (infected leaves/total leaves per plant) was also significantly lower ($p < 0.05$) on LA2747 and LA458 in comparison with

Moneymaker, where 33.3, 29.6 and 59.4% were recorded respectively (Table 5.1). The first leaves on LA2747 and LA458 were dead after 8 days (2.0 and 1.5 leaves per plant) where the heavy inoculum of the mildew was present (Plates 5.3 and 5.4) whereas only 0.3 dead leaves resulted on Moneymaker (Table 5.1). Dead leaves were significantly lower ($p < 0.01$) on Moneymaker in comparison to LA2747 and LA458. The mildew colonies covered more than 50% of Moneymaker foliage compared to 5-10% of LA2747 and LA458 foliage (Table 5.1). Disease symptoms were significantly higher on Moneymaker leaves ($p < 0.001$) compared to the wild lines LA2747 and LA458. After 4 weeks of infection heavily infected leaves of Moneymaker died off starting from the older leaves but there was no leaf shedding, while new growth from axils replaced the dead leaves on LA2747 and LA458 plants (Plates 5.5 and 5.6).

Table 5.1: Screening of tomato cv. Moneymaker and two wild lines (LA458 and LA2747) against powdery mildew

Tomato Genotype	Colonies per Plant	Disease Incidence	Dead Leaves per Plant	Disease Score
Moneymaker	516.5 a	59.4 a	0.30 b	5.00 a
LA458	50.3 b	33.3 b	2.00 a	1.50 b
LA2747	68.8 b	29.6 b	1.50 a	1.80 b

- Values within a column followed by the same letter are not significantly different at 5% level by Duncan's Multiple Range Test.
- Four plants from each line were inoculated with conidia from diseased plants and incubated at 24 ±1°C, 16 hours photoperiod, 180-250 $\mu E\ m^{-2}\ s^{-1}$ and 88-92% rh.
- Disease symptoms were assessed with the following scale:
0 = No symptoms; 1 = 1-4% of the foliage surface covered with mildew; 2 = 5-10% of the foliage surface covered with mildew; 3 = 11-25% of the foliage surface covered with mildew; 4 = 26-50% of the foliage surface covered with mildew; 5 = More than 50% of the foliage surface covered with mildew.

Plates 5.3-6: Screening of wild lines of tomato (LA2747and LA458) and tomato cv. Moneymaker (susceptible) against powdery mildew

Plate 5.3: Screening of wild lines of tomato (LA2747 and LA458) and tomato cv. Moneymaker (susceptible) against powdery mildew 10 days after inoculation

Plate 5.3a: Moneymaker and LA2747

Plate 5.3b: Moneymaker and LA458

Symptoms of powdery mildew developed on the lower 4-5 leaves of tomato cv. Moneymaker (MM) and heavily infected leaves remained on the plants, but fewer leaves of the wild lines LA2747 and LA458 showed the disease symptoms and the heavily infected leaves desiccated.

Note the difference in the morphology of the wild lines and Moneymaker (MM) plants


5.3-A
MM
Many maker
LA 2743


5.3-B
MM
Many maker
LA 458

Plate 5.4a: Powdery mildew symptoms on the third leaf of tomato cv. Moneymaker (MM)

Plate 5.4b: Powdery mildew symptoms on the third leaf of tomato line LA2747 Note the difference in leaf shape between the two genotypes and the extent of powdery mildew coverage was less on LA2747 compared to cv. Moneymaker


5.4-A


5.4-B

Plate 5.5-6: Screening of wild lines of tomato (LA 458) and cv. Moneymaker (MM) against powdery mildew 4 weeks after inoculation

Plate 5.5: Moneymaker (MM) and LA458 plants 4 weeks after inoculation with powdery mildew

Plate 5.6: Moneymaker (MM) and LA2747 plants 4 weeks after inoculation with powdery mildew
Powdery mildew symptoms extended much further up plants of cv. Moneymaker and older leaves dried out, but there was no leaf abscission. On the wild genotypes of tomato the disease remained confined to lower inoculated leaves and new growth developed from the axils of these dried leaves


LA 458
M M
5.5


LA 2747
M M
5.6

This indicated the wild lines have some resistance to powdery mildew shown phenotypically as rapid cell death (hypersensitivity) on the heavy infected lower leaves, level of disease infection was 1.5 compared to 5.0 on Moneymaker (Table 5.1). Leaves of wild lines were smaller, hairier and tougher than the leaves of Moneymaker (Section 5.3.5).

5.1.5 Scanning Electron Microscopy of Powdery Mildew on Leaves of Tomato Lines

Powdery mildew was investigated with scanning electron microscopy to compare the fungal structures on the leaf surface of the wild lines of tomato (LA2747 and LA458) and Moneymaker. Leaf discs (4 mm diameter) from the terminal leaflet of the third leaf were taken 12 days after inoculation of plants, fixed, dehydrated, dried in a critical point dryer and coated with gold before examination with the scanning electron microscope (section 2.6.4).

The fungus produced only a single conidium at the end of each conidiophore (Plate 5.7). Conidia germinated on the leaf surface forming short germ tubes terminating in a forked appressoria (Plate 5.8). Subsequently, secondary elongating hyphae were formed. On Moneymaker leaves, the fungus became well established and formed a dense network of secondary hyphae that spread to cover much of leaf area whilst LA2747 and LA458 had less conidia that germinated and developed appressoria (Plates 5.9-11). A relatively sparse fungal development was also shown on leaves of LA2747 and LA458 in comparison with cv. Moneymaker (Plates 5.12-14). The fungus penetrated the leaves of the three genotypes of tomato directly through the cuticle and no penetration through the stomates was observed (Plates 5.15-17). Leaf hairs are evenly distributed on the leaf surfaces of LA2747 and LA458 and shorter compared to the leaf hairs of Moneymaker which are longer and densely concentrated on the mid ribs (Plates 5.18-20).

Plates 5.7-20: Scanning Electron Microscopy

All samples were taken 12 days after inoculation of leaves

Plate 5.7: Powdery mildew conidiophore on tomato cv. Moneymaker 12 days after inoculation. The conidiophore is septate and a single terminal conidium is formed

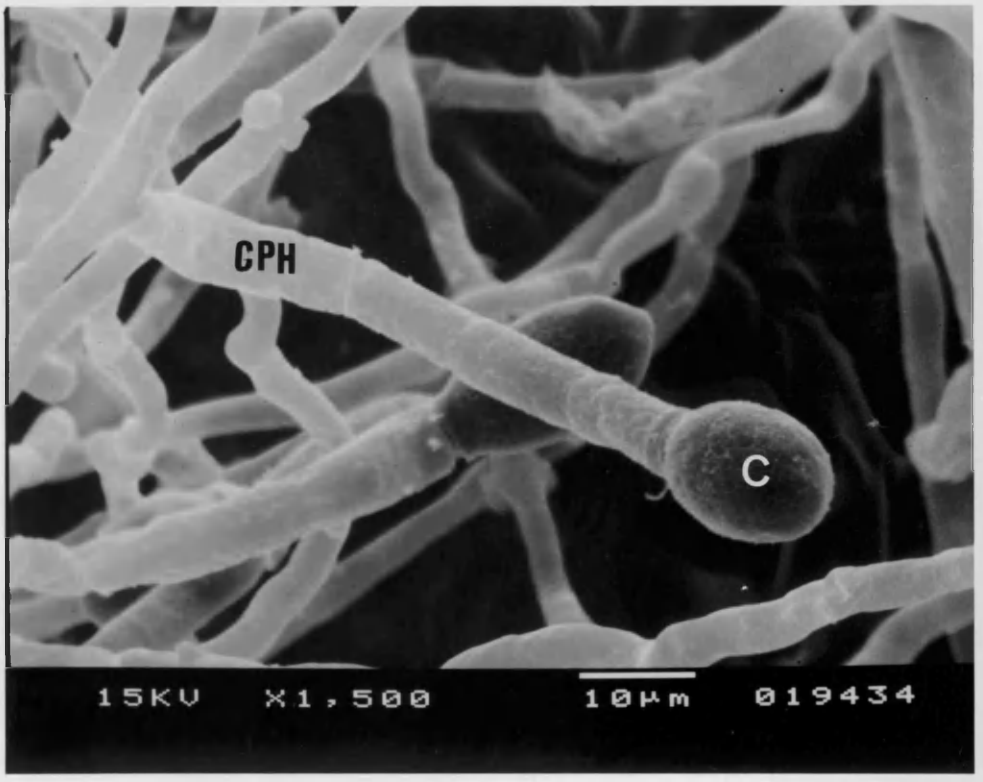
Plate 5.8: Lobate appressorium arising from a conidium on tomato line LA458.

C Conidium

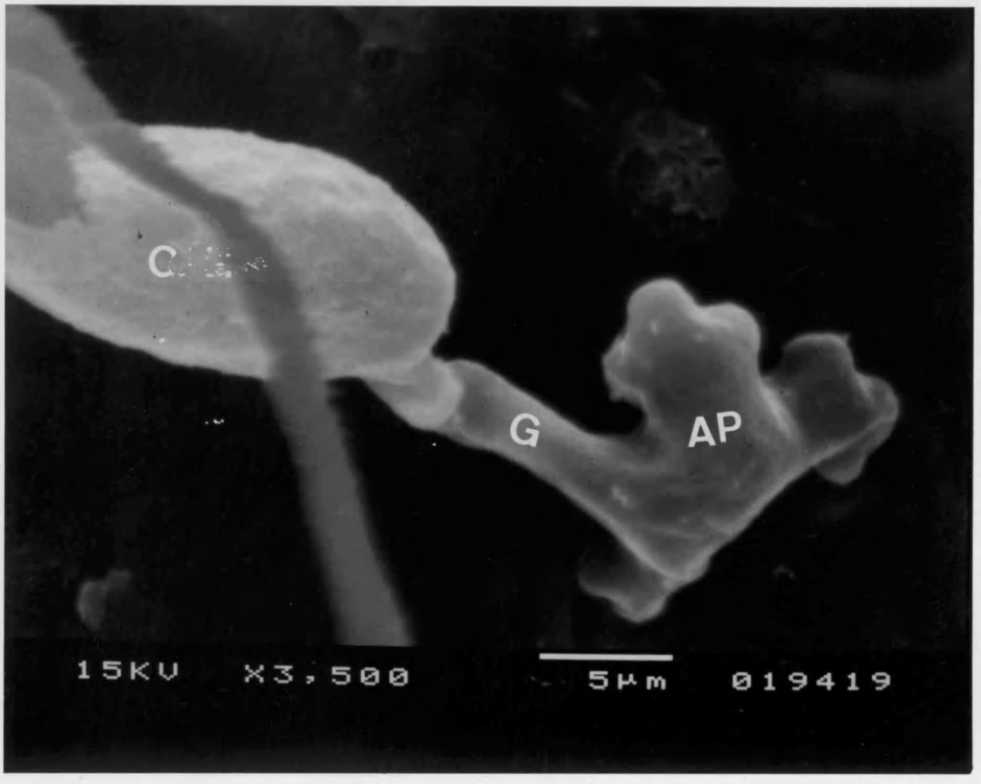
G Germ tube

AP Appressorium

CPH Conidiophore



5.7



5.8

Plate 5.9: Development of powdery mildew on the leaf surface of tomato cv. Moneymaker. A dense network of powdery mildew conidiophores has developed on the leaf surface

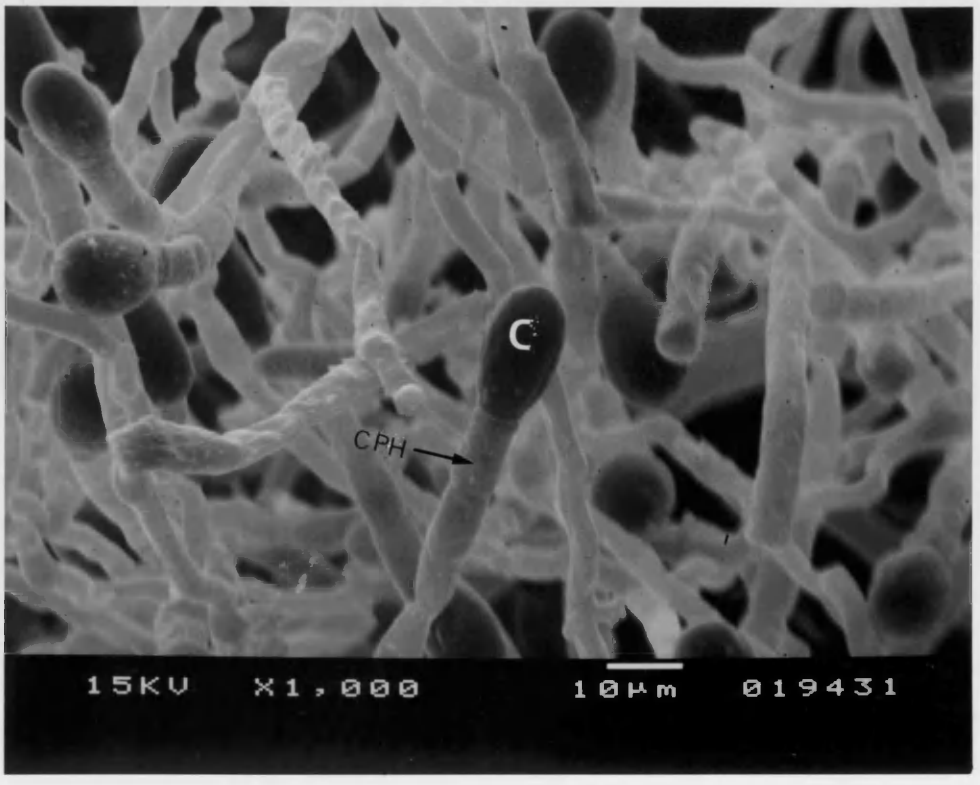
Plate 5.10: Germinated conidia on the leaf surface of tomato line LA2747. Note the terminal and intercalary lobed appressoria

C Conidium

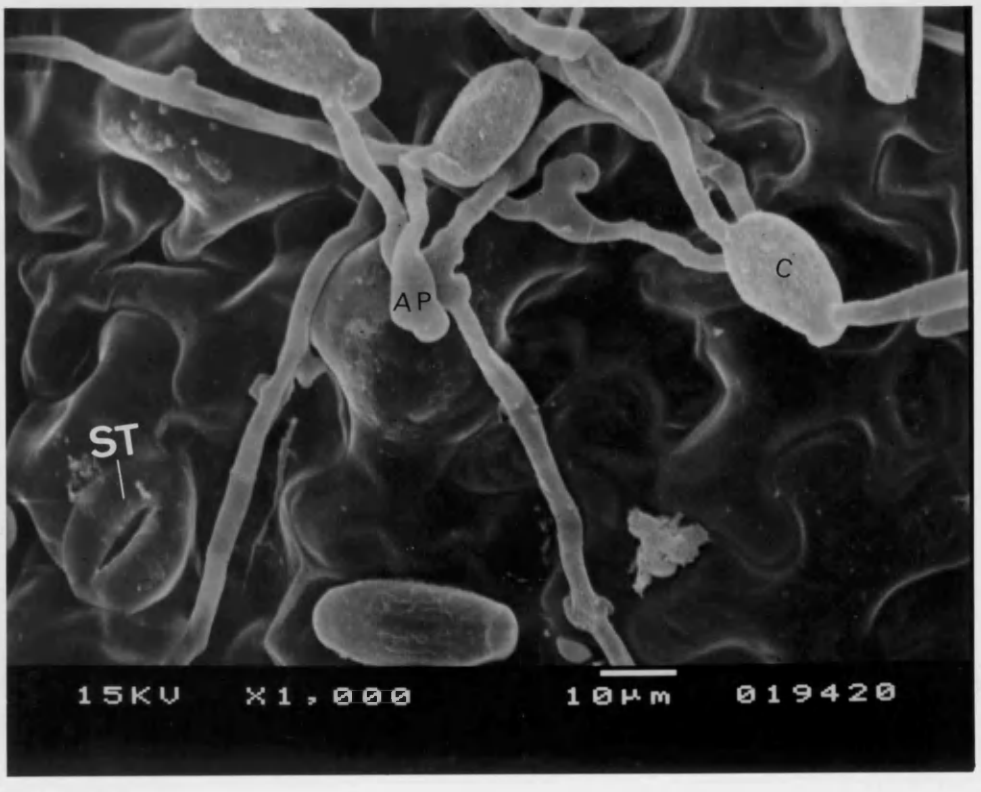
ST Stomate

AP Appressorium

CPH Conidiophore



5.9



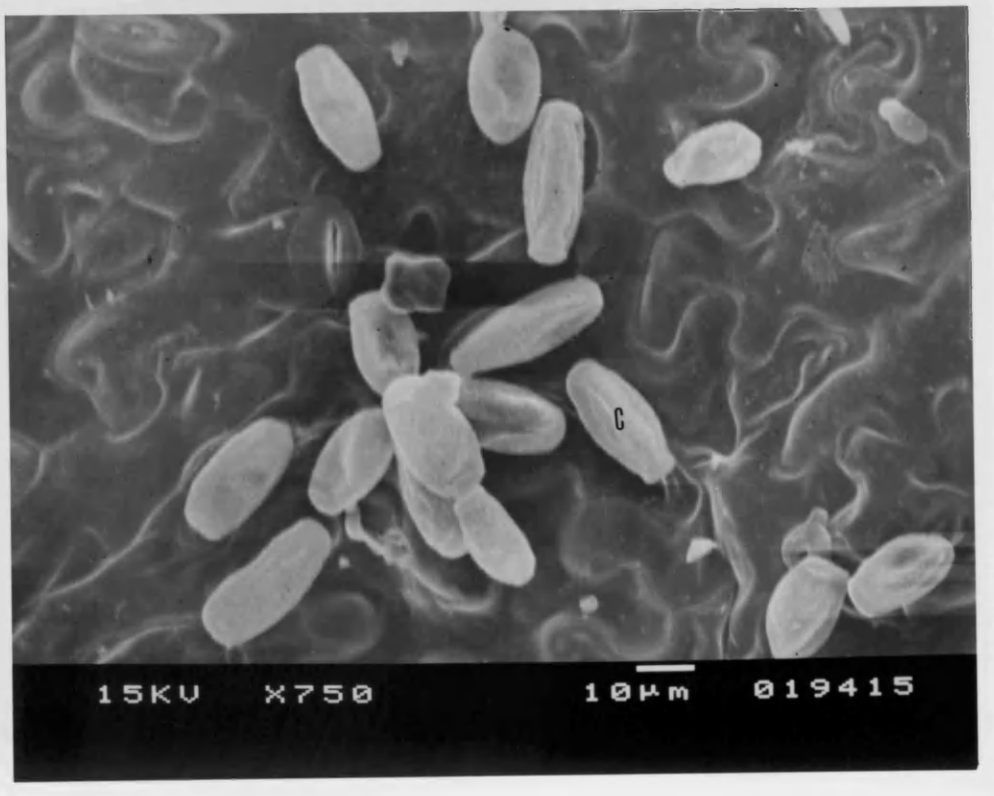
5.10

Plate 5.11: Low germination of powdery mildew conidia on the leaf surface of the tomato line LA458

Plate 5.12: Extensive development of powdery mildew mycelium and conidia on tomato cv. Moneymaker

C Conidium

CPH Conidiophore



5.11

5.12

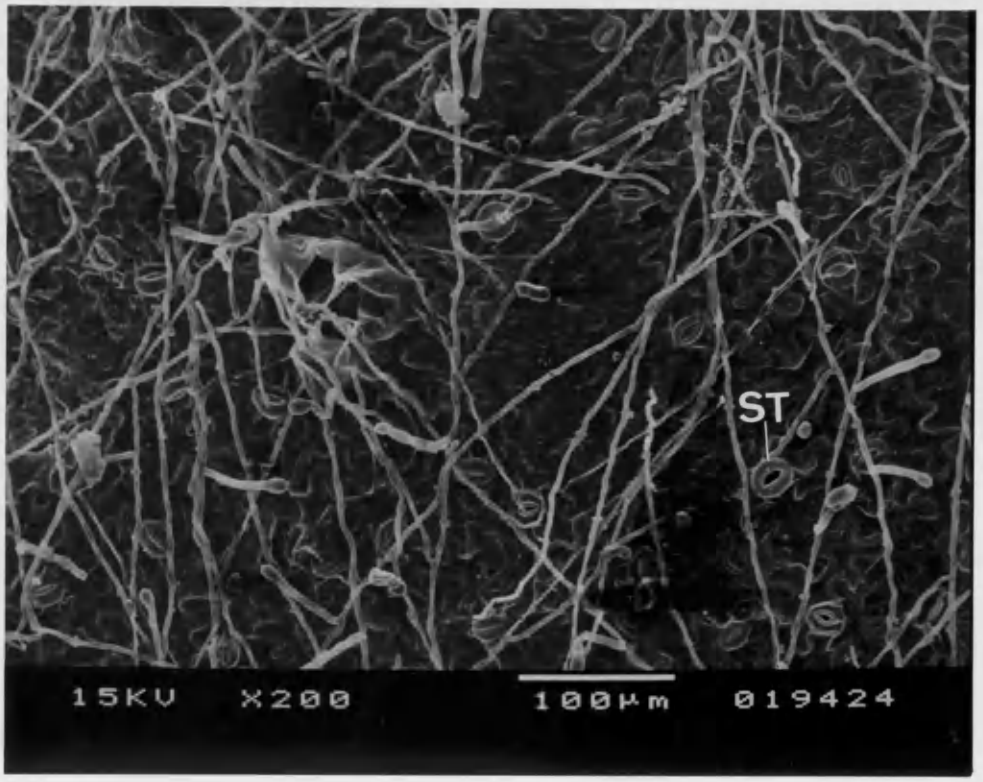
Plate 5.13: Powdery mildew on the leaf surface of tomato line LA2747. Note the sparse sporulation

Plate 5.14: Sparse development on the leaf surface of the tomato line LA458. Some germinated conidia have formed appressoria. A few germinated conidia have formed secondary hyphae. Contrast to Plate 5.12.

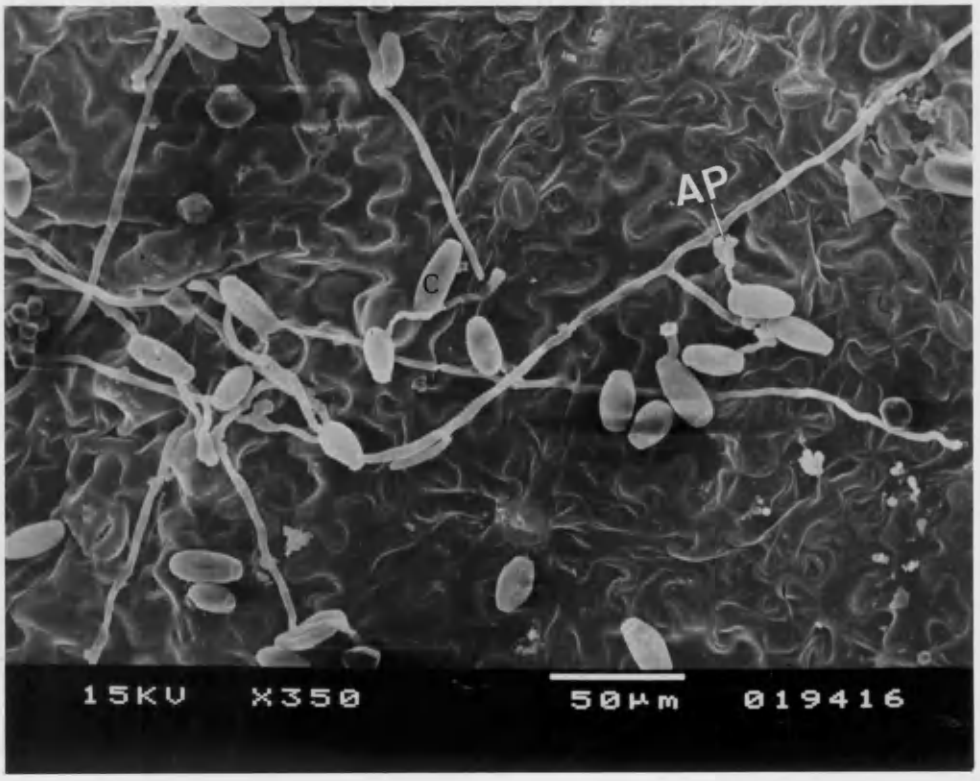
C Conidium

ST Stomate

AP Appressorium



5.13



5.14

Plate 5.15: Detail of conidium formation and penetration into tomato cv. Moneymaker leaf. Penetration was directly through the leaf epidermis via an appressorium

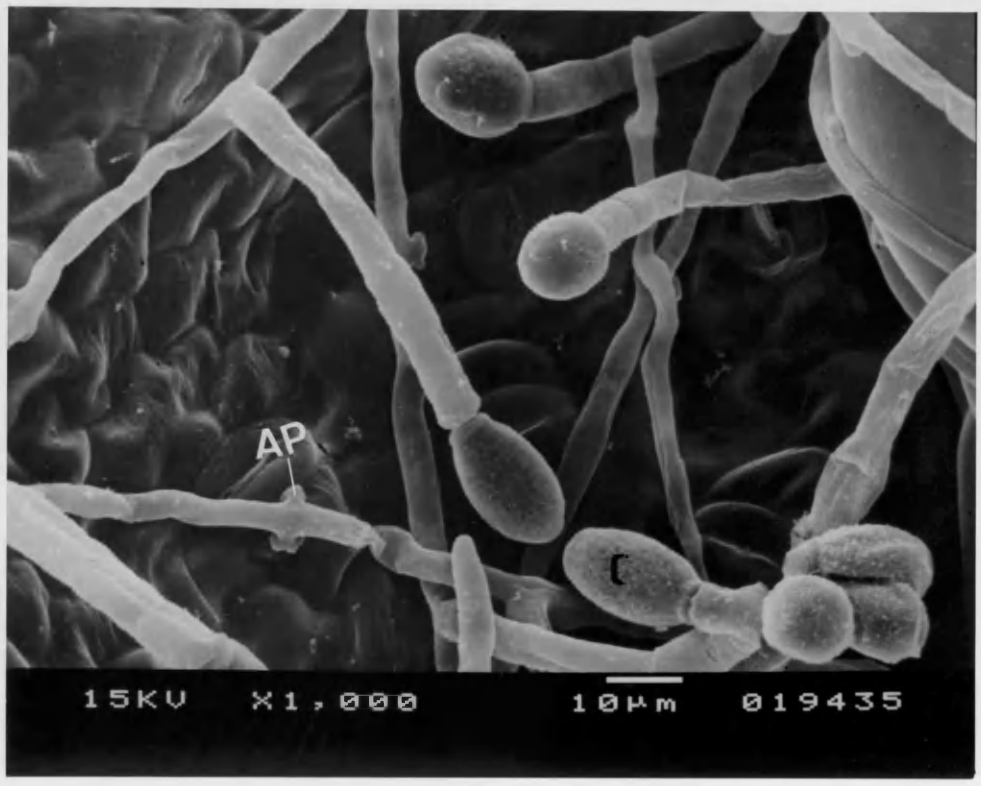
Plate 5.16: Powdery mildew on tomato LA2747 leaf surface. Fungal structures formed are similar to those in Plate 15 and penetration also appears to be directly through the leaf epidermis

C Conidium

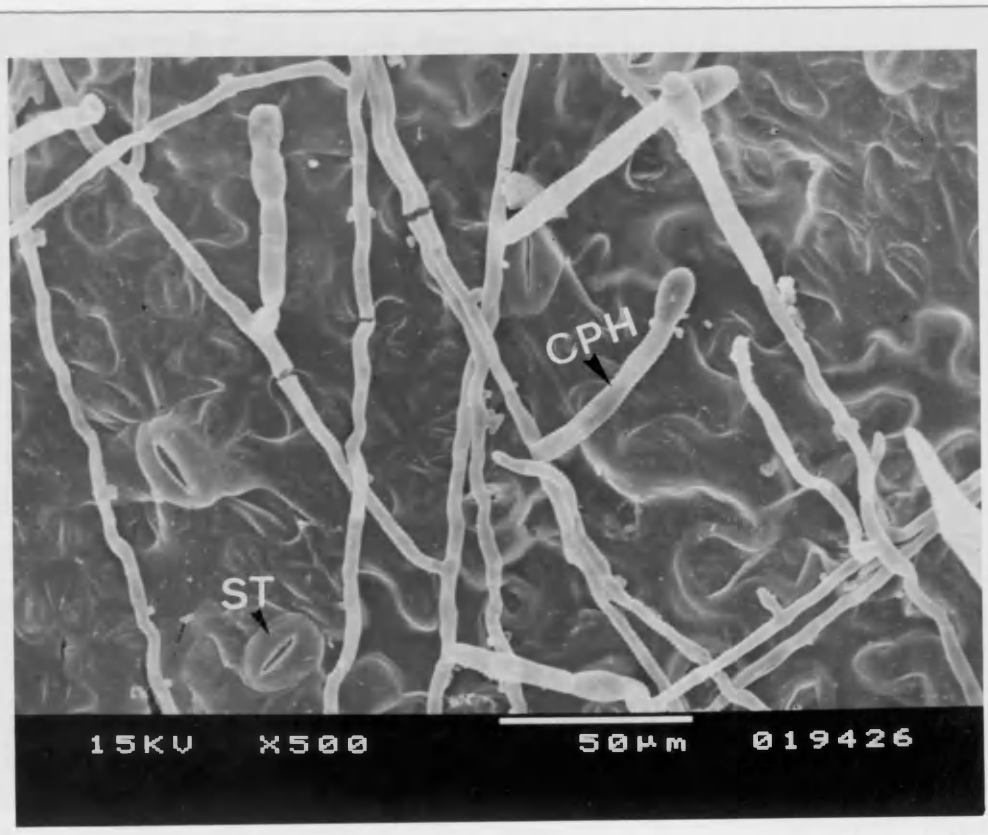
ST Stomate

AP Appressorium

CPH Conidiophore



5.15



5.16

Plate 5.17: Appressoria arising along hyphae on LA458 leaf surface

Plate 5.18: Powdery mildew on the leaf surface of tomato cv. Moneymaker leaf. Note the long leaf hairs densely arranged on the midrib and the coarse topography of leaf surface

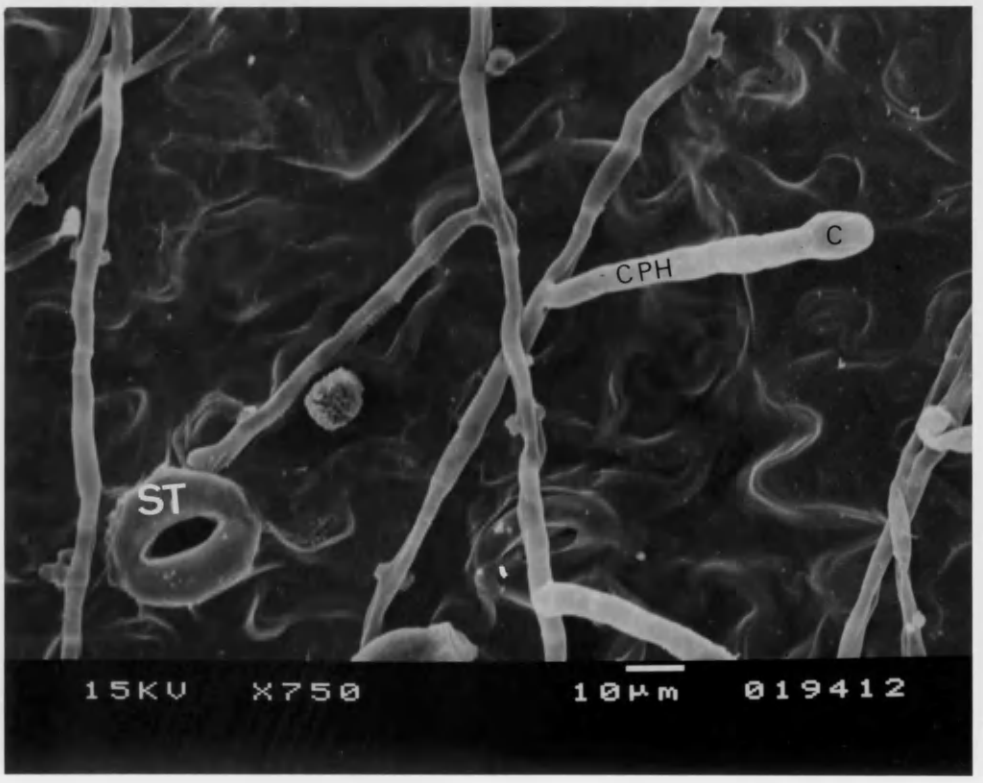
C Conidium

S Secondary hypha

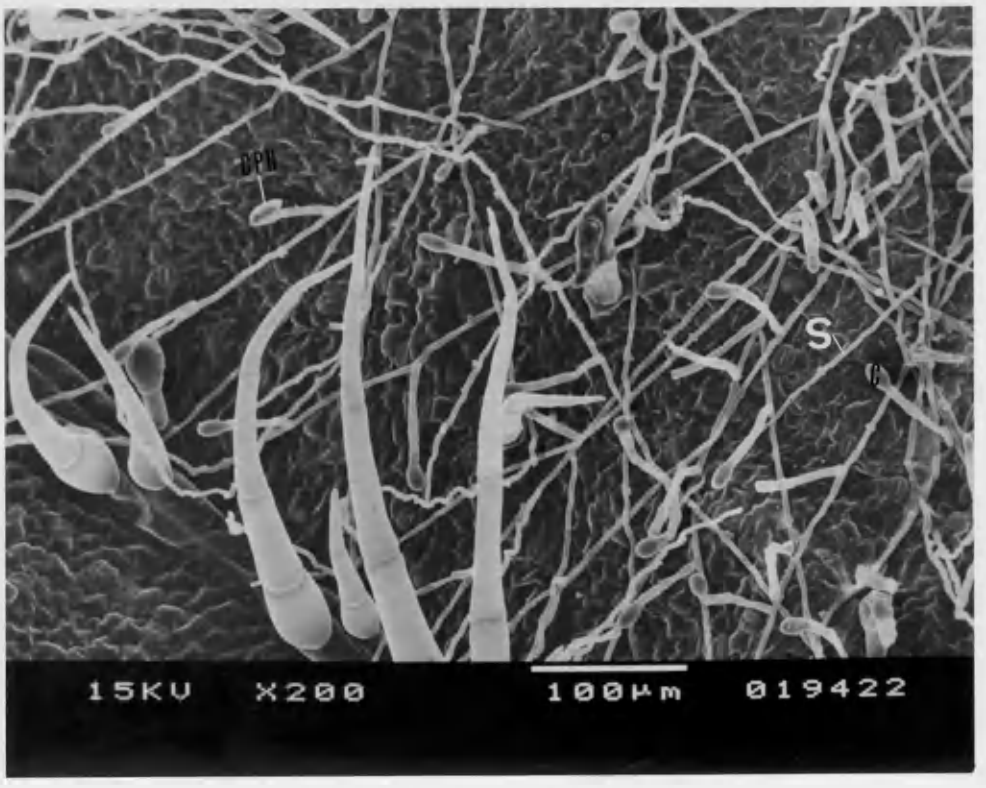
ST Stomate

AP Appressorium

CPH Conidiophore



5.17



5.18

Plate 5.19: Powdery mildew on the leaf of tomato line **LA458** and the leaf topography which is smoother with evenly distributed leaf hairs throughout the leaf surface. The leaf hairs were shorter with 2 septa compared to 3-4 septa of Moneymaker leaf hairs

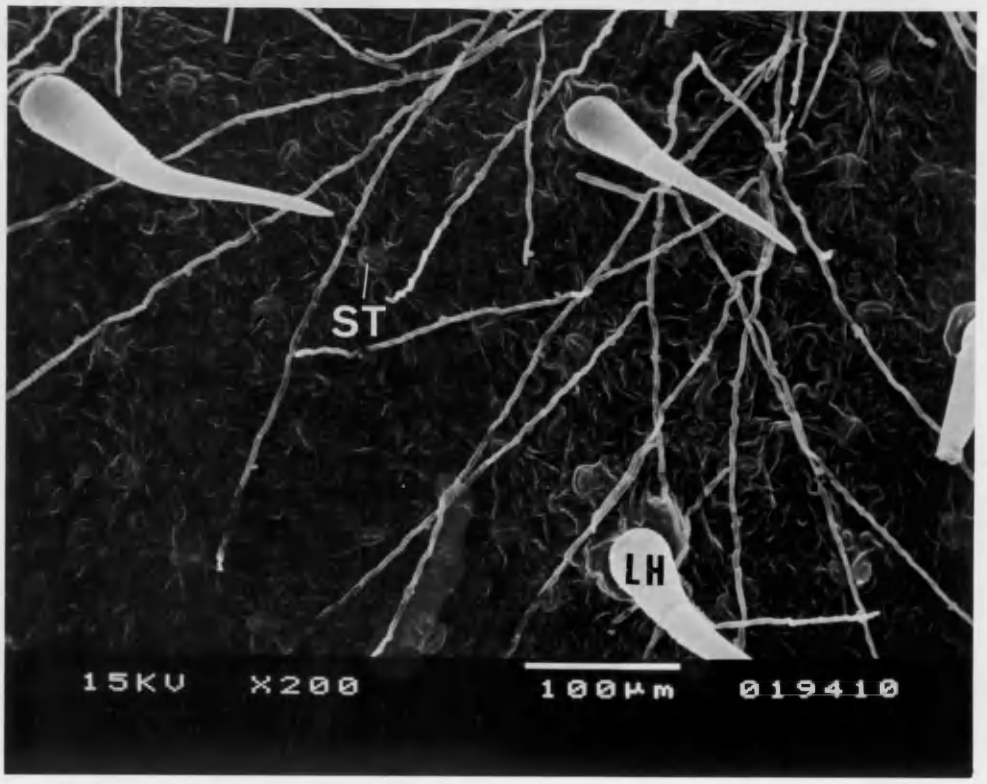
Plate 5.20: Powdery mildew on leaves of tomato line **LA2747.** Fungal structures and leaf topography were as in plate **5.19, but the leaf hairs have** been damaged during preparation.

S Secondary hypha

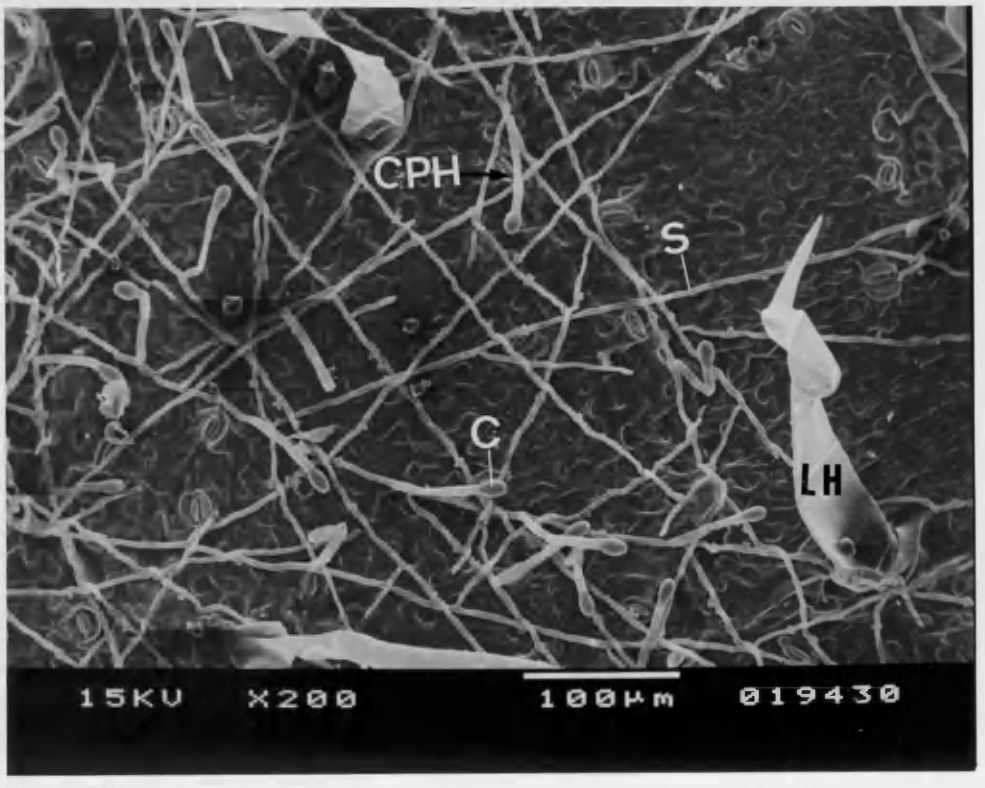
C Conidium

CPH Conidiophore

LH Leaf hair



5·19



5.20

5.1.6 Light microscopy of Mildew Structures on Tomato Leaves

The low conidial germination observed by SEM on leaves of LA2747 and LA458 needed further confirmation as well as an investigation of factors that might contribute to the resistance of these lines. Thus germination of conidia on leaf discs from the three tomato genotypes was compared under carefully controlled conditions.

5.1.6.1 Germination of Conidia on Leaf Discs of Moneymaker and the Wild Tomato Genotypes (LA2747 and LA458) *In Vitro*

Leaf discs (9 mm diameter) were excised from Moneymaker, LA2747 and LA458 laid adaxial surface up on moistened filter paper beds to keep the humidity high. Petri dishes (5 cm diameter) containing the leaf discs were inoculated with conidia from infected plants using a settling tower (Section 2.6.1). Then the petri dishes were sealed and incubated at 25 °C, 16 hours photoperiod and 50 μE $m^{-2}s^{-1}$ light. After 24 hours leaf discs were then stained by a droplet of calcofluor (0.01%) and examined under UV light without a coverslip with an Olympus BH-2PC fluorescent microscope set up as in Section 2.6.4. Fungal hyphae and conidia fluoresced blue, and plant cells remained dark or emitted only a weak red autofluorescence. Stomates, leaf hairs and physically injured cells also autofluoresced blue. Only isolated conidia were counted to avoid self inhibition which may occur in groups. One hundred conidia were observed for germination and 25 conidia for germ tube lengths. The experiment was repeated three times with consistent results. Because the inoculum was collected from infected plants kept in the greenhouse at minimum temperature 24 °C (section 2.2.1), the germination was relatively low in the three experiments because upper limit of the temperature and rh were not controllable in the greenhouse. The germination of conidia produced under this condition, was considerably affected.

The germination was found to be significantly different ($p < 0.001$) between the three genotypes. It was 82.91, 67.11, 37.6 and 35.81 on glass, Moneymaker, LA2747 and LA458 discs respectively (Figure 5.8a). The conidial germination on glass was significantly higher than germination on tomato leaf discs of all the genotypes. Conidial germination on the leaf discs of LA2477 and LA458 were similar, and there was significantly less than germination on Moneymaker leaf discs. Germ tubes were significantly longer ($p < 0.001$) on glass than on Moneymaker discs, which was also significantly lower on LA458 and LA2747 (Figure 5.8b); germ tube lengths on the 2 wild types was similar.

5.1.6.2 Internal and External Structures of Powdery Mildew in Leaf Discs of Moneymaker, LA2747 and LA458

For the observation of internal structures of the powdery mildew leaf discs were inoculated with conidia and incubated for three days to allow formation of fungal infection structures. The leaf discs were then fixed on paper moistened with alcoholic glacial acetic acid, cleared and softened by lactophenol (section 2.6.3), then stained by calcofluor and examined under UV light for conidial germination, penetration and haustorium formation (section 2.6.4). Epidermal strips removed with forceps, were also examined after staining by calcofluor.

Plates 5.21 and 5.22 show the low germination of powdery mildew conidia on LA2747 and LA458 compared to that on Moneymaker leaf discs (Plate 5.23). Conidia that germinated on Moneymaker discs developed short germ tubes, appressoria and secondary hyphae. After 3 days of incubation, elongation of the secondary hyphae was a clear indicator of successful infection (Plate 5.24) but no haustoria were seen. There were few cases (< 5%) in which germinated conidia developed appressoria or secondary hyphae on leaves of wild lines. Affected epidermal cells in LA2747 and LA458 autofluoresced yellow under the appressoria or hyphae (Plates 5.25 and 5.26) perhaps as a sign of a hypersensitive

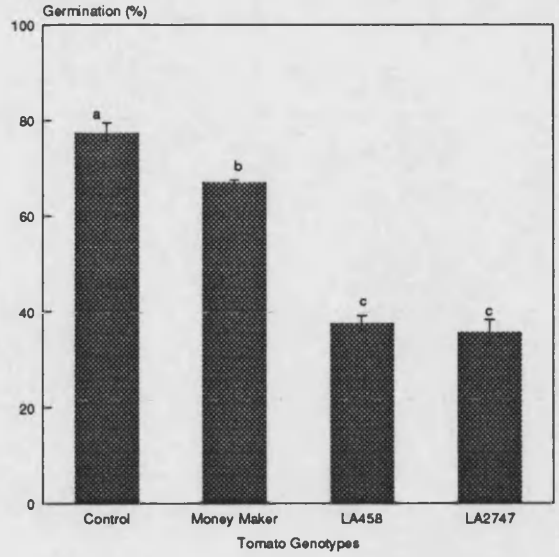


Figure 5.8a: Germination of powdery mildew conidia on leaf discs of different tomato genotypes

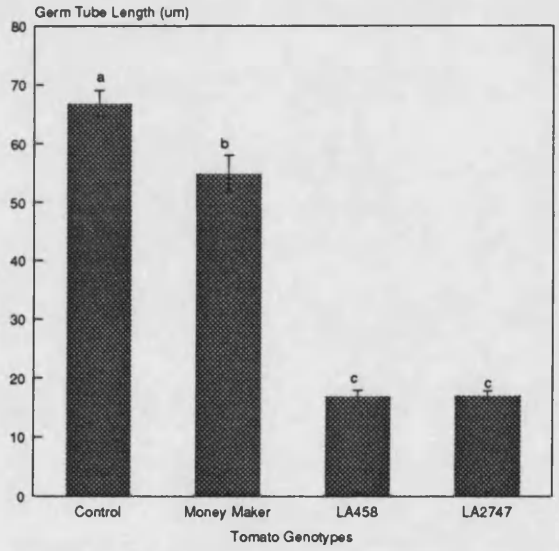


Figure 5.8b: Elongation of powdery mildew germ tubes on leaf discs of different tomato genotypes

Inoculated leaf discs were incubated at 25 °C, 16 h day, 100% rh and light intensity 50 µE m-2 s-1. Cover slips were used as a control treatment. Hundred conidia and 25 germ tube were assessed per replicate. Data are the means and standard errors of 3 replicates.

Plates 5.21-23: Light microscopy of germination of powdery mildew conidia on leaf discs of tomato cv. Moneymaker and the wild genotypes LA458 and LA2747 3 days post inoculation.

These leaf discs were stained by calcofluor and examined under UV light

Plate 5.21: Low germination of powdery mildew conidia on the leaf disc of the tomato genotype LA2747. Note the shrunken size of the conidia.

Bar = 20 μ

Plate 5.22: Low germination of powdery mildew conidia on leaf disc of the tomato genotype LA458.

Bar = 20 μ

Plate 5.23: Relatively higher germination of powdery mildew conidia on the leaf disc of tomato cv. Moneymaker. The inoculum was from plants grown in a greenhouse (minimum temperature 25 °C) which resulted in lower germination (68%) compared to the conidia collected from a growth cabinet used in the rest of the experiments which was ≤ 90%.

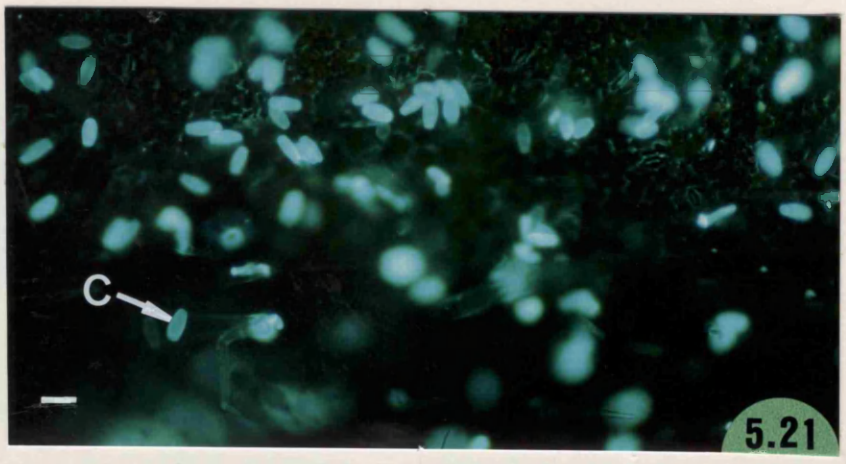
Bar = 20 μ

C Conidium

AP Appressorium

S Secondary hypha

G Germ tube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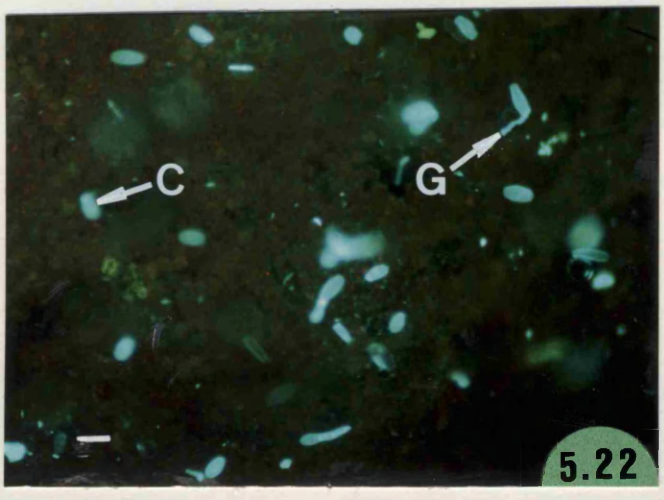
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G
5.22

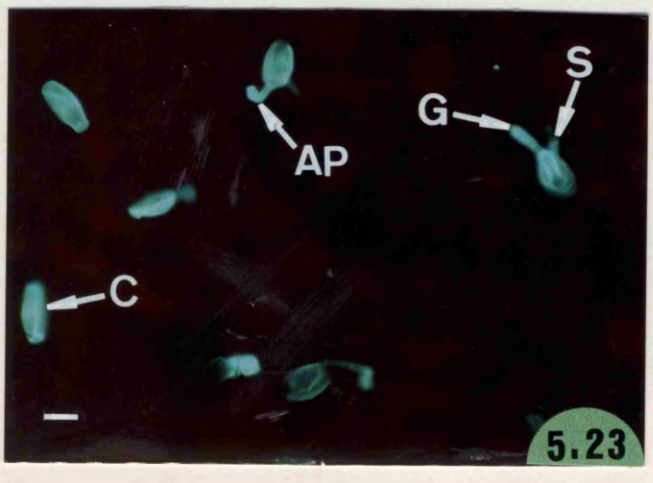
S
G
AP
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Plate 5.24-27: Interaction of powdery mildew with the tomato genotypes LA2747 and LA458 in comparison with Moneymaker 3 days after inoculation

Plate 5.24: Elongating secondary hypha(e) on leaf disc of tomato cv. Moneymaker 3 days after inoculation

Bar = 5 μ

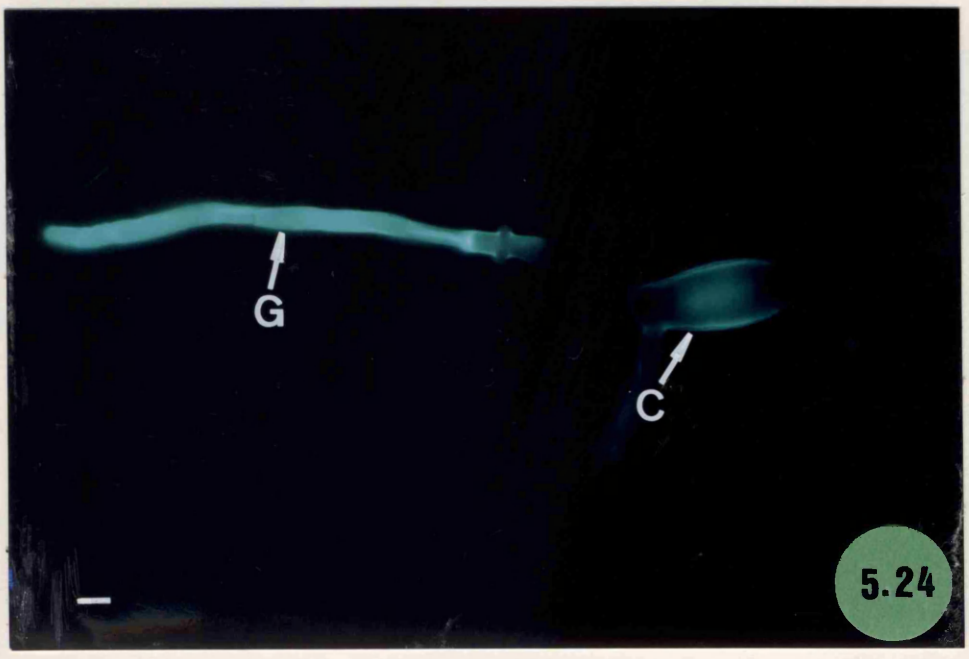
Plate 5.25: Apparent hypersensitive reaction beneath powdery mildew appressorium in the tomato line LA2747.

Bar = 20 μ

C Conidium

G Germ tube

AP Appressorium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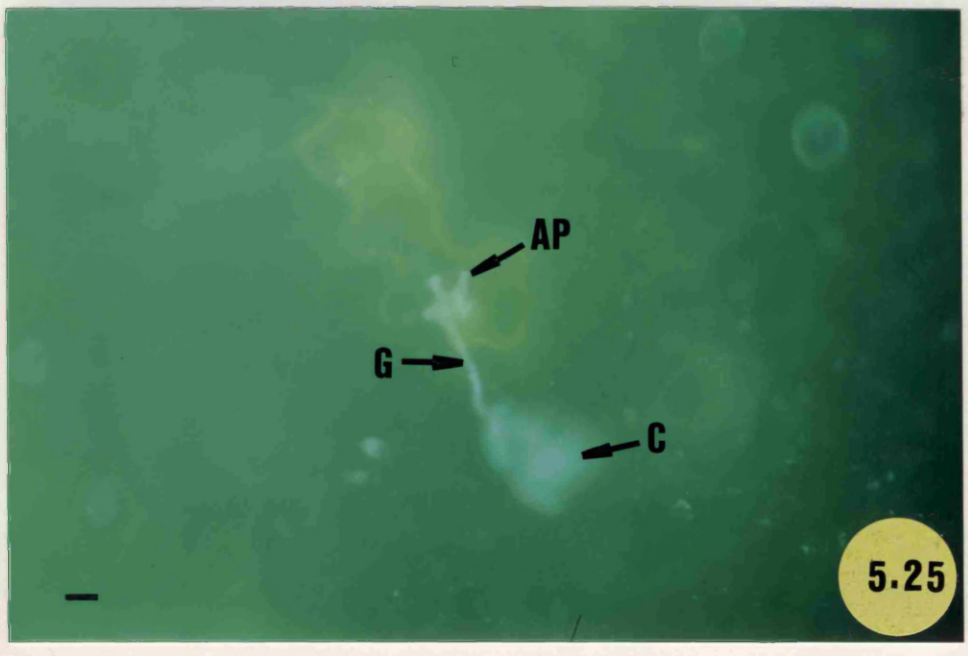
AP
G
C
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Plate 5.26: Two hypersensitive cells in leaf disc of tomato LA458 line; the cells appear to be associated with infection structures from a single conidium.

Bar = 20 μ

Plate 5.27: Germination and appressorium formation on cv. Moneymaker. No autofluorescence in host cells was associated with any appressoria or germ tubes.

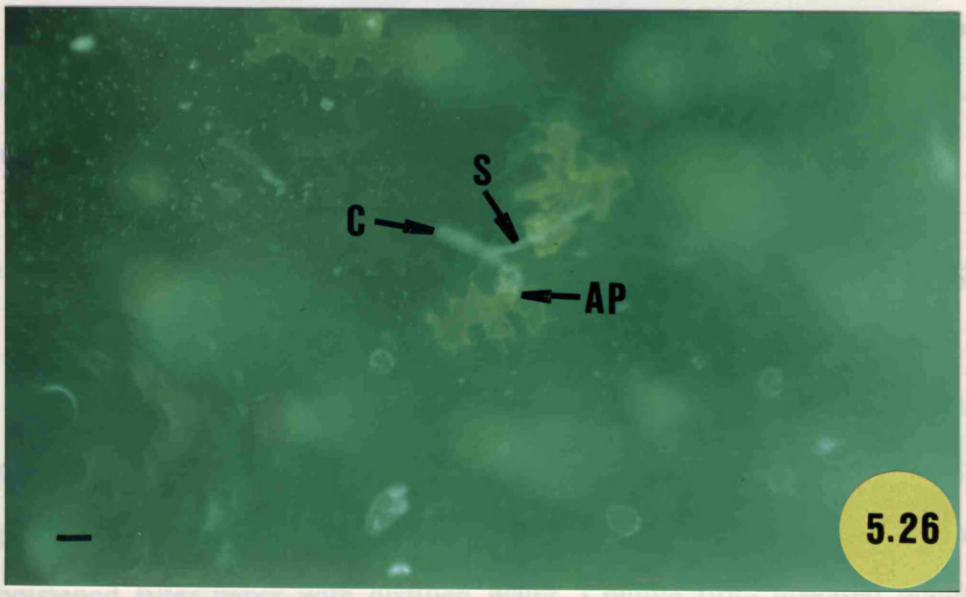
Bar = 10 μ

C Conidium

G Germ tube

AP Appressorium

S Secondary hypha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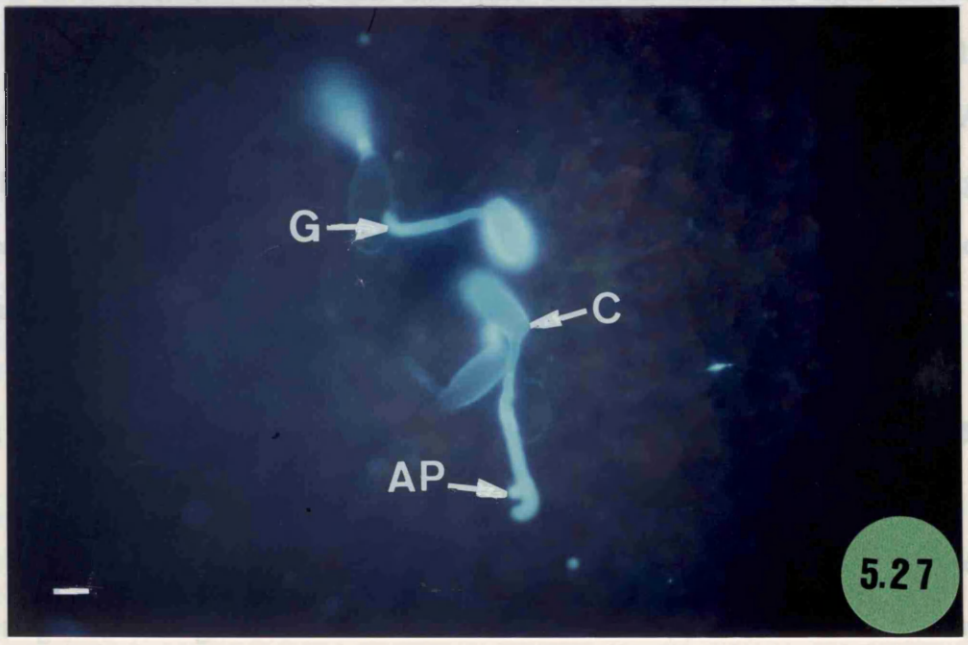

G
C
AP
5.27

reaction, but no equivalent change was observed in infected areas on Moneymaker (Plate 5.27). Also the cleared leaf discs showed dark brown stained areas in the epidermis of LA458 and LA2747 (Plates 5.28 and 5.29), but no stained areas were observed in the epidermis of Moneymaker (Plate 5.30). The discoloured areas were fewer but larger in LA458 compared to LA2747. In addition LA2747 had more lateral veins.

5.1.7 Effect of Volatiles Produced by Leaves from Moneymaker, LA458 and LA2747 on the Germination of Powdery Mildew Conidia

The low germination of conidia on leaf discs of wild lines could be a result of an inhibitor on or in these leaves. To test for a possible volatile inhibitor(s) 10 g of leaves from each genotype were placed in petri dish (9 cm diameter) on moistened filter paper with distilled water. Conidia from diseased plants were collected on coverslips (18 X 18 mm) and the coverslips were then cemented to the lids of the petri dishes with Vaseline (Figure 2.1). Control chambers differed by the abscence of leaf tissue. After 24 hours the germination and germ tube lengths in 3 replicates from each line were observed under the light microscope.

There was no difference between all the treatments and the control. The germination was 93.9, 96.4, 93.3 and 93.1% in the control, Moneymaker, LA2747 and LA458 respectively (Figure 5.9a). Germ tubes were significantly longer in the control compared to petri dishes containing leaves of the 3 tomato genotypes (Figure 5.9b).

5.1.8 Effect of Water and Chloroform Washings from Leaves of Moneymaker, LA2747 and LA458 on Conidia Germination

From the previous work it was apparent that no volatile substance was affecting conidial germination. To test for inhibitor(s) associated with the leaf surface 10 g of undamaged leaves from Moneymaker, LA2747 and LA458 were

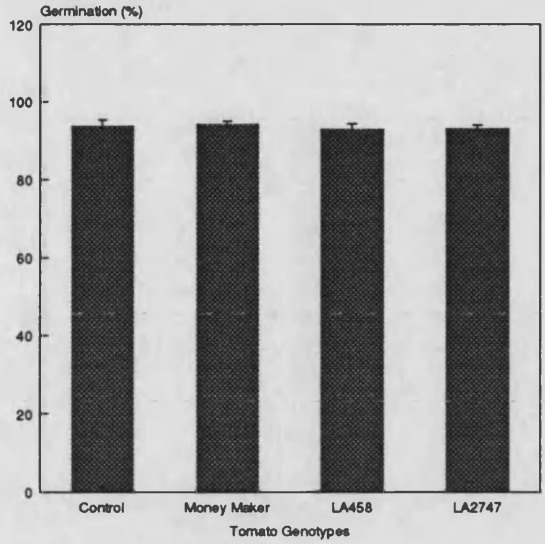


Figure 5.9a: Attempts to detect a volatile inhibitor from tomato leaves; germination of powdery mildew conidia on glass incubated with leaves of different tomato genotypes

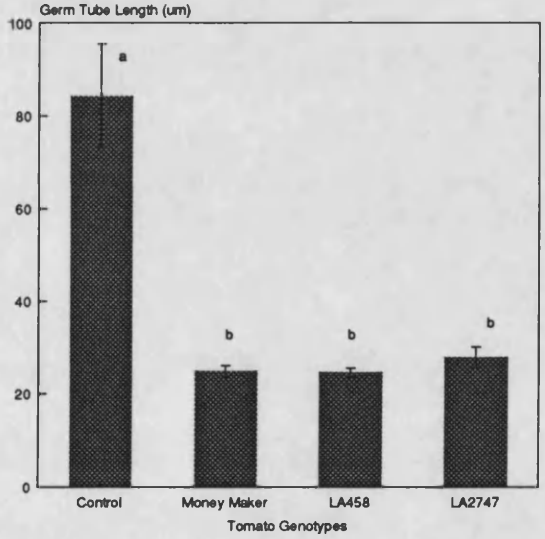


Figure 5.9b: Attempts to detect a volatile inhibitor from tomato leaves; elongation of powdery mildew germ tubes on glass incubated with leaves of different tomato genotypes

The test was done at 25° C, 16 h day, 100% rh and light intensity 50 µE m-2 s-1. Germination of 25o conidia and length of 25 germ tube per replicate were assessed 24 h after incubation. Data are the means and standard errors of 3 replicates.

Plate 5.28-30: Cleared leaf discs of tomato cv. Moneymaker and the genotypes LA458 and LA2747 3 days after inoculation. Adaxial surface

Plate 5.28: Line LA458. Note the large glandular leaf hairs.

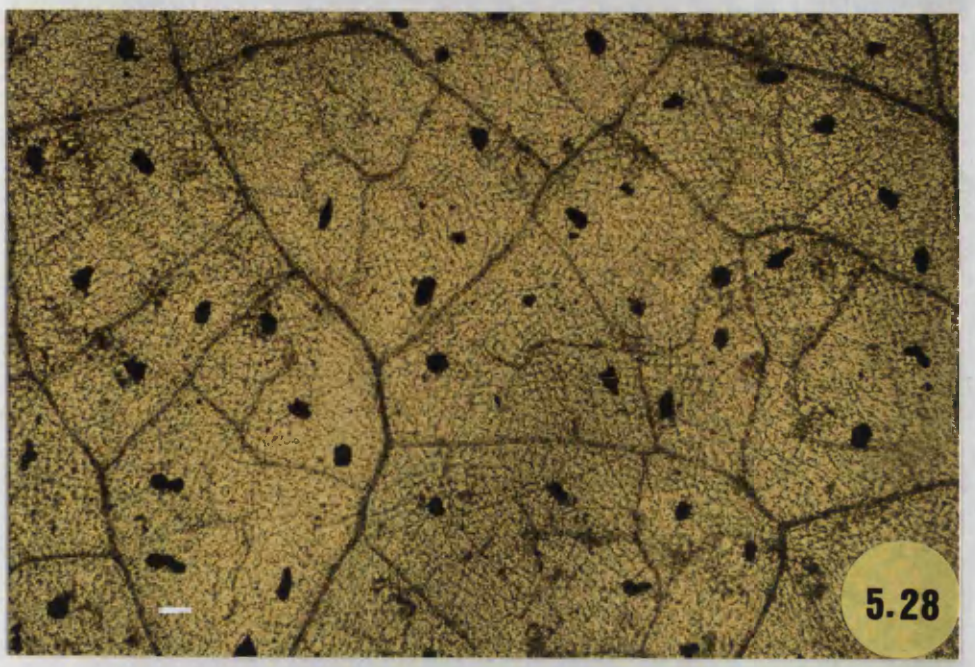
Bar = 100 μ

Plate 5.29: Line LA2747. Note the more frequent, smaller glandular hairs and and extensive branching of leaf veins.

Bar = 100 μ

Plate 5.30: cv. Moneymaker. Note the absence of leaf hairs and the larger interveinal areas.

Bar = 100 μ



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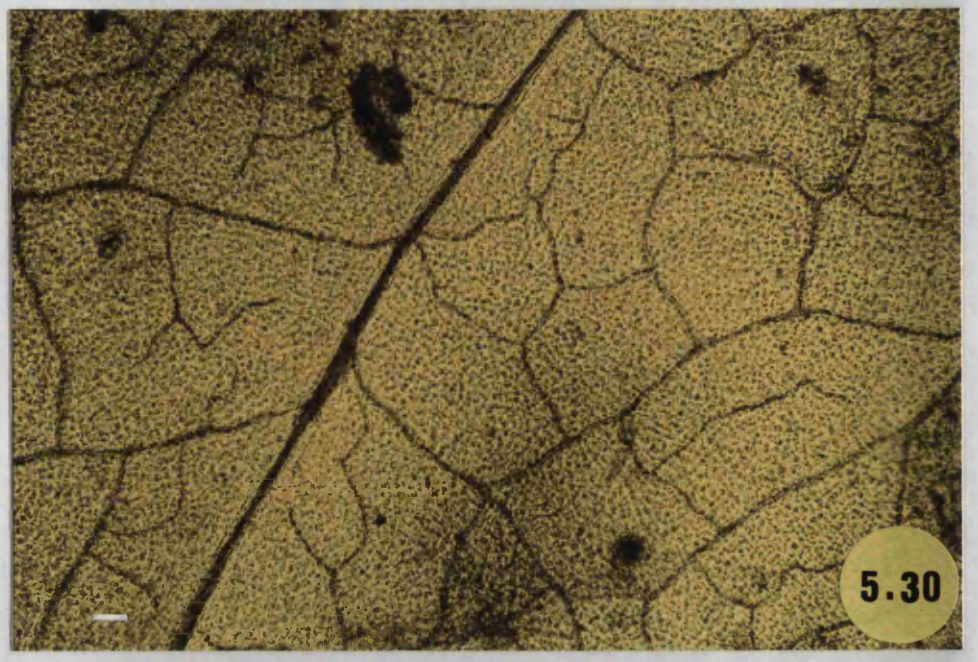
5.29



5.30

washed with 10 ml of either chloroform or water for 5 minutes. The washes were then filtered by filter paper and dried under reduced pressure in a rotary evaporator. The dried samples were then redissolved into 2 ml of either chloroform or water respectively, distributed over the whole area and left to dry on clean cover slips. These coverslips were then inoculated with conidia from infected tomato plants and incubated in a humidity chamber with moistened filter paper. Water or chloroform were dried on coverslips as controls. Three replicates of each treatment were observed for conidial germination after 24 h. Angular transformation (arcsine) was used before the statistical analysis of the data and means were separated by Duncan Multiple Range Test.

High germination (≥ 85%) was achieved with both controls. The germination of conidia was significantly higher ($p < 0.001$) in the control (water dried on the coverslips) compared to the other leaf washes of the three genotypes (Figure 5.10) which were similar in reducing germination from 88.7% for the control down to 30.5-34.2%. Similarly, germinated conidia on the chloroform washes of Moneymaker, LA2747 and LA458 were significantly lower than the control ($p < 0.001$, Figure 5.11). Conidial germination on Moneymaker chloroform wash was significantly higher (about 20 fold) than those from the wild genotypes of tomato LA2747 and LA458 but there was no difference in germination on extracts from the 2 wild lines (Figure 5.11 and Plates 5.31a-d) which remained at only 25%.

Thus, there appears to be a chloroform soluble inhibitor(s) of germination associated with the leaf surface either unique to or at a higher levels in the wild genotypes.

5.1.9 Screening of Tomato Genotypes against Powdery Mildew

Breeder lines of tomato as well as tomato somaclones (SC_1) and their progenies (SC_2) from *in vitro* culture of Moneymaker were screened against powdery mildew. The plants were inoculated with conidia from infected tomato

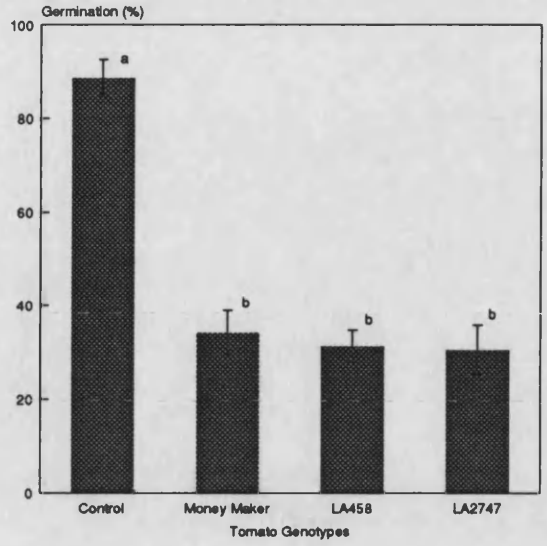


Figure 5.10: Attempts to detect a germination inhibitor from the surface of tomato leaves; germination of powdery mildew conidia on glass covered with water washes from leaves of different tomato genotypes

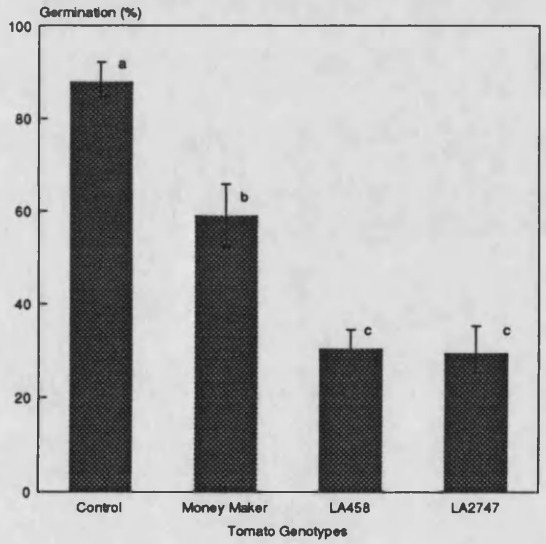


Figure 5.11: Attempts to detect a germination inhibitor from the surface of tomato leaves; germination of powdery mildew conidia on glass covered with chloroform washes from leaves of different tomato genotypes

The germination was tested at 25° C, 16 h day, 100% rh and light intensity 50 µE m-2 s-1. Cover slips were used as a control treatment. the germination of 100 conidia was assessed per replicate. Data are the means and standard errors of 3 replicates.

Plate 5.31a-d: Conidial germination on glass covered with chloroform washes from leaves of different genotypes of tomato

Plate 5.31: Germination of powdery mildew conidia on glass covered with chloroform washes from different tomato genotypes at 25 ^{0}C and 100% relative humidity

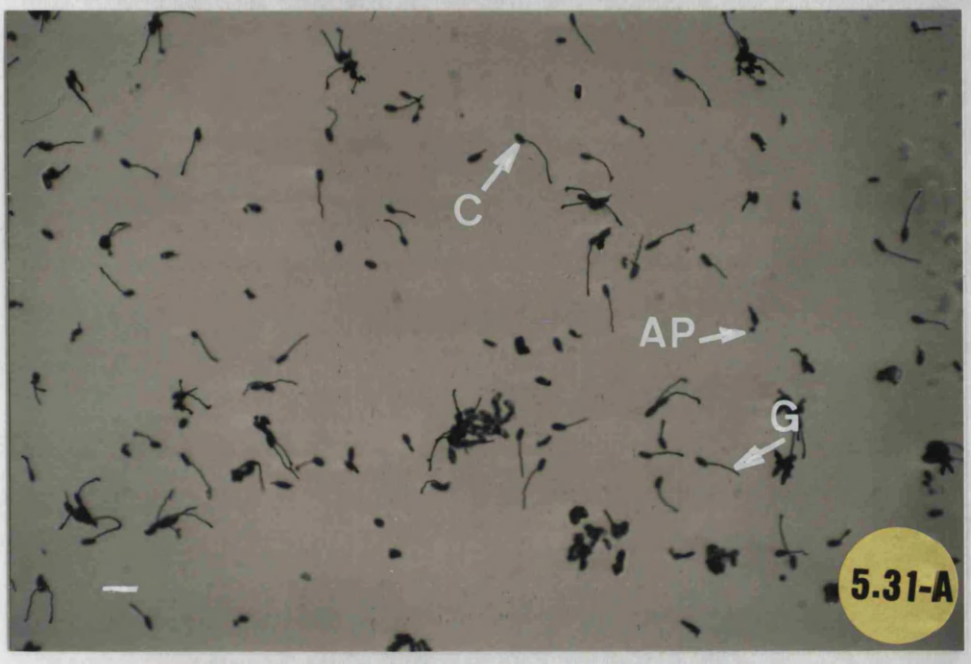
Bar = 100 μ

Plate 5.31a: Conidial germination in the chloroform (control).

Plate 5.31b: Germination of powdery mildew conidia on chloroform washes of leaves of cv. Moneymaker. Note the reduced germination compared to the control.

Plate 5.31c: Germination of powdery mildew conidia on chloroform washes of leaves of LA458 line. Note the low germination and the deposition of chloroform soluble material, probably cuticular wax.

Plate 5.31d: Germination of powdery midew conidia on the chloroform washes of LA2747 line. Details as in Plate 31.c.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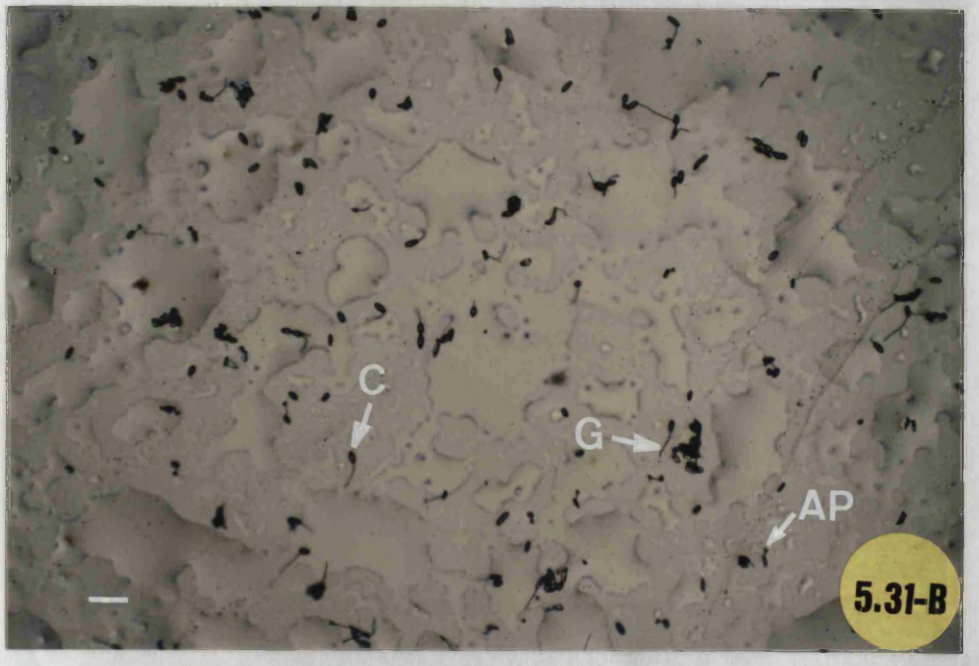
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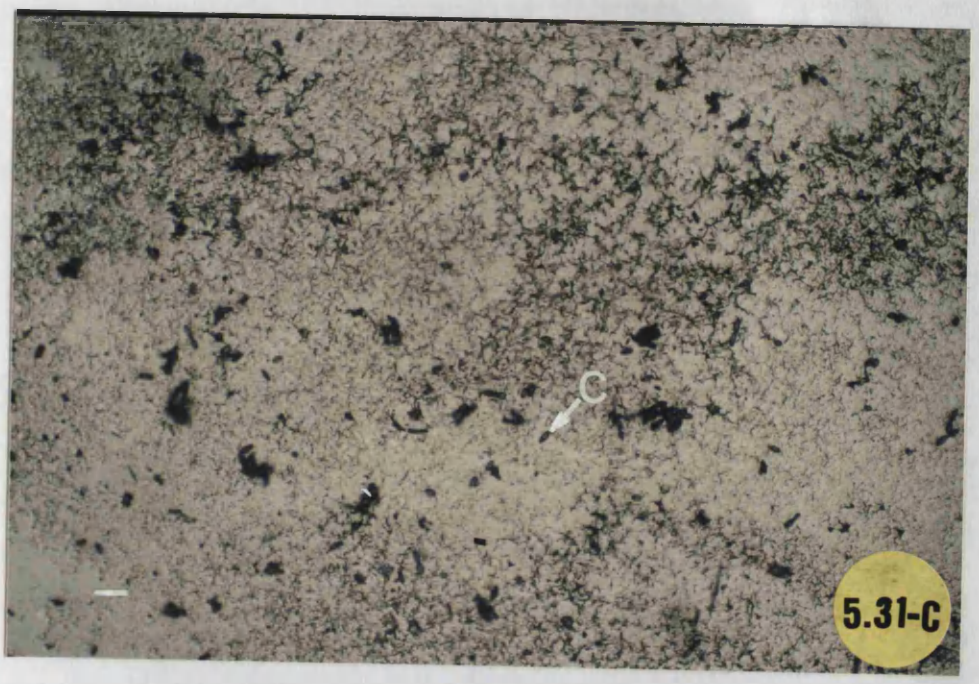
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5.31-C


C
5.31-D

plants, and incubated in a Fisons growth chamber (Section 2.6.1) for one week and then the number of colonies per inoculated leaf were counted and divided by the area of the infected leaves. Disease incidence (No. of leaves showing disease symptoms / total No. of inoculated leaves) was also assessed and the plants were scored for disease symptoms according to scale from 0 to 5 as in Section 5.3.4

Tomato lines from a breeding programme for resistance against tomato leaf curl virus (TLCV) were also selected for their partial resistance against powdery mildew in the field (Geneif, 1983). These lines were crosses between Moneymaker or strain B with the *L. pimpinellifolium* line LA1478 which has monogenic resistance to TLCV. The breeder lines SLC 73, 70, 58 and 44 are progenies of Moneymaker X LA1478 whereas SLC 26, 27, 28, and 29 are progenies of Strain B X LA1478. Five plants of each line and of the two tomato cultivars were screened against powdery mildew.

The lines varied in the number of mildew colonies per 10 mm^2 of leaf area, infection index and disease symptoms. The lines which had a Moneymaker (indeterminate) background were more susceptible to powdery mildew than those coming from strain B (determinate) crosses. Strain B progenies had 8.2 to 10.6 colonies per 10 mm^2 compared to 7.7 to 12.4 for those of Moneymaker progenies (Table 5.2). On all strain B progenies two out of the three inoculated leaves developed the disease symptom after one week, but all the inoculated leaves of Moneymaker progenies developed severe mildew symptoms (Disease score 5) whereas the disease score on strain B progenies was 4.

The somaclones were regenerated from Moneymaker explants and cloned. Three clones from each plant were screened against the powdery mildew and the results showed that some lines had reduced number of colonies per 10 mm^2 leaf area, disease incidence and symptoms score, but there was no clear resistance expressed in these lines (Table 5.3). Other lines were even more susceptible than

Table 5.2: Screening of tomato breeder lines against powdery mildew

Tomato breeder line	Mildew colonies per 10 mm^2 leaf area	Disease incidence (%)	Disease score
SLC 73	12.36 g	100	5
SLC 70	11.88 f	100	5
SLC 58	7.72 a	100	5
SLC 44	11.72 f	100	5
SLC 29	10.64 e	66.7	4
SLC 28	8.84 c	66.7	4
SLC 27	8.22 b	66.7	4
SLC 26	9.76 d	66.7	4
strainB	9.12 c	100	5
Money maker	15.88 h	100	5

Five plants from each line were inoculated with conidia from diseased plants and incubated at 24 ±1°C, 16 hours photoperiod, 180-250 µE m^{-2} s^{-1} and 88-92% rh.

Disease symptoms were assessed with the following scale:
0 = No symptoms; 1 = 1-4% of the foliage surface covered with mildew; 2 = 5-10% of the foliage surface covered with mildew; 3 = 11-25% of the foliage surface covered with mildew; 4 = 26-50% of the foliage surface covered with mildew; 5 = More than 50% of the foliage surface covered with mildew.

Values within a column followed by the same letter are not significantly different at 5% level according to Duncan's Multiple Range Test.

Tables 5.3 and 5.4 : Screening of Tomato Somaclones (Sc1) and Their Progenies against Powdery Mildew

Four plants from each line were inoculated with conidia from diseased plants and incubated at 24 ±1°C, 16 hours photoperiod, 180-250 $\mu E\ m^{-2}\ s^{-1}$ and 88-92% rh.

Disease symptoms were assessed with the following scale:
0 = No symptoms; 1 = 1-4% of the foliage surface covered with mildew; 2 = 5-10% of the foliage surface covered with mildew; 3 = 11-25% of the foliage surface covered with mildew; 4 = 26-50% of the foliage surface covered with mildew; 5 = More than 50% of the foliage surface covered with mildew.

Original source of explants:

HY	: Hypocotyl
SI	: Stem Internode
C	: Cotyledon
L	: Leaf
S	: Seed

Values within a column followed by the same letter are not significantly different at 5% level according to Duncan's Multiple Range Test.

Table 5.3: Screening of somaclones (Sc_1) regenerated from tomato cv. Moneymaker explants

Tomato somaclone (Sc_1)	Original source of explant	Mildew colonies per 10 mm^2 leaf/area	Disease incidence	Disease score
1	SI	11.4 x	100	5
2	L	16.5 no	100	5
3	SI	17.4 m	100	5
4	L	11.9 w	100	5
5	SI	10.8 y	100	5
6	SI	16.2 o	100	5
7	SI	12.1 w	100	5
8	SI	16.0 o	100	5
9	C	13.6 t	100	5
10	C	16.6 n	100	5
11	C	10.2 y	100	5
12	C	11.3 x	100	5
13	C	22.6 i	100	5
14	C	15.5 p	100	5
15	C	15.5 p	100	5
16	C	12.3 w	100	5
17	C	12.9 uv	100	5
18	C	14.3 s	100	5
19	C	14.9 qr	100	5
20	SI	13.7 t	100	5
21	C	8.7 z	100	5
22	HY	12.8 v	100	5
23	HY	12.9 uv	100	5
24	C	12.8 v	100	5
25	C	12.0 w	100	5
26	C	11.2 x	100	5
27	HY	11.8 w	100	5
28	YH	16.8 n	100	5
29	HY	20.2 k	100	5
30	L	32.6 b	100	5
31	SI	15.5 p	100	5
32	L	13.4 tu	100	5
33	L	11.2 x	100	5
34	HY	21.4 k	100	5
35	L	28.1 d	100	5
36	C	15.4 pq	100	5
37	SI	24.7 fg	100	5
38	SI	19.7 k	100	5
39	HY	26.4 e	100	5
40	HY	24.4 g	100	5
41	HY	25.1 f	100	5
42	HY	22.3 i	100	5
43	HY	23.4 h	100	5
44	HY	26.8 e	100	5
45	C	30.5 c	100	5
46	HY	14.7 p	100	5
47	SI	33.1 a	100	5
48	SI	16.8 n	100	5
49	HY	22.3 i	100	5
Moneymaker	S	18.0 l	100	5

Moneymaker. Seeds were collected from these somaclones and screened for resistance against powdery mildew; in spite of a significant variation in the number of mildew colonies, no resistant line was found (Table 5.4).

5.2 Discussion

The percentage germination of conidia *in vitro* and the elongation of germ tubes are the most widely used criteria for the determination of optimum temperature of powdery mildews (Clerk and Ayesu-offei, 1967; Manners and Hossain, 1963; Yarwood et al., 1954; Caesar and Clerk, 1985; Nour, 1958). The effects of temperature and rh on the germination and germ tube elongation were investigated here. Light intensity and photoperiod were reported to have little or no effect on the germination of conidia of powdery mildew (Ward and Manners, 1974; Nair and Ellingboe, 1965). Germination and germ tube elongation of *Erysiphe cichoracearum* were found to occur at a wide range of temperature and humidity. These results correspond with those of Manners and Hossain (1959) on *E. graminis* and Correll (1986) on *Leveillula taurica*. The optimum temperature for the germination of *E. graminis* f. sp. (*avenae, hordei, tritici*) reported by Manners and Hossain (1959) was 20 °C, and the germination of conidia decreased above or below this temperature. In support of the result found in this study Yarwood et al. (1954) reported that the optimum and maximum temperatures for *E. cichoracearum* were about 22 °C and 34 °C respectively, but at the minimum temperature (9 °C) reported by these authors for this fungus, no germination of conidia was observed in this study at any humidity level. Based on 13 independent records Yarwood et al. (1954) reported that the average minimum, optimum, and maximum temperatures for *E. graminis* were 3, 17, and 31 °C respectively. In contrast Nair and Ellingboe (1965) suggested that due to physiological differences between spores in the population of *E. graminis* f. sp. *tritici*, the germination of these spores was better when they were incubated under

Table 5.4: Screening of tomato somaclones progenies (Sc_2) against powdery mildew

Progeny of tomato somaclone (Sc_2)	Original source of explant	Mildew colonies per 10 mm^2 leaf area	Disease incidence	Disease score
1	SI	22.5 de	100.0	5.0
2	SI	25.8 a	100.0	5.0
3	SI	22.7 de	73.3	5.0
4	SI	21.6 g	66.7	4.6
5	SI	14.4 rs	100.0	5.0
6	HY	18.2 ij	93.3	5.0
7	HY	12.4 vw	80.0	4.0
8	HY	21.1 g	93.3	5.0
9	L	17.4 jk	93.3	5.0
10	L	18.4 i	100.0	4.4
11	C	14.1 st	80.0	5.0
12	C	13.6 t	100.0	5.0
13	C	16.7 lm	100.0	5.0
14	C	14.8 st	100.0	4.6
15	C	18.3 i	80.0	5.0
16	C	18.5 i	100.0	5.0
17	C	12.2 w	80.0	5.0
18	C	14.5 rs	100.0	5.0
19	C	13.6 t	93.3	5.0
20	C	22.1 f	100.0	5.0
21	C	13.8 t	100.0	5.0
22	HY	14.0 st	100.0	5.0
24	YH	15.9 o	100.0	5.0
25	L	14.9 q	100.0	5.0
26	L	16.3 n	100.0	5.0
27	L	22.7 d	100.0	5.0
28	L	20.1 h	100.0	5.0
29	HY	18.2 ij	100.0	5.0
30	L	25.7 a	100.0	5.0
31	SI	16.5 mn	100.0	5.0
32	L	13.1 u	100.0	5.0
34	L	17.3 kl	100.0	5.0
35	HY	15.5 p	100.0	5.0
36	L	24.5 b	100.0	5.0
37	C	13.8 t	100.0	5.0
38	SI	12.7 v	100.0	5.0
40	SI	22.2 ef	100.0	5.0
41	HY	15.6 o	100.0	5.0
42	HY	15.6 o	100.0	5.0
Moneymaker	S	24.9 b	100.0	5.0

two environmental conditions instead of one. They found that successive incubation for 1 hr at 17 °C and 100% rh followed by 3 hr at 22 °C and 65% rh gave high germination. These results suggested that the use of single conidia with uniform age as well as the control of environmental conditions under which the germination was investigated are very important for consistency of results. Nour (1958) attributed the low germination of crowded conidia of *L. taurica* to the possible production of CO_2 from the respiration of conidia which acts as a germination inhibitor. A self inhibitory effect was also reported on crowded conidia by Domsch (1954) (reviewed in Nour (1958)). In contrast, Clerk and Ayesu-offei (1967) reported that at 30 °C and 100% rh the conidia of *L. taurica* in chains of any length germinated as freely as single spores and the position of the conidium has no effect on its germination potential. Clerk and Ayesu-offei (1967), Nour (1958) and Correll (1986) reported lower percentage germination of *L. taurica* conidia compared to the germination of those of *E. cichoracearum* in this study. These differences may have been due to the difference in age of conidia, the environment under which conidia were produced, or the solution used for the control of humidity level. Clerk and Ayesu-offei (1967) used conidia from plants that were grown in a greenhouse and shaken every 6 days, in addition they used H_2SO_4 instead of NaOH used in this study. Caesar and Clerk (1985) reported the possibility of the reduction of germination of conidia of *L. taurica* due to a small amount of SO_2 gas which can evolve from H_2SO_4 used for the control of humidity. The effect of the environment under which conidia were produced was reported by Ward and Manners (1974) on conidia of *E. graminis*. They found that germinability and infectivity of conidia were highest in conidia produced at 20 °C and at high rh.

The complex effect of temperature and humidity on the germination and germ tube elongation was also observed by Manners and Hossain (1959). In this study the temperature optimum for germ tube elongation was found to be slightly

higher than the optimum for conidial germination which corresponds with results of Manners and Hossain (1959). The implication of these results on the development of powdery mildew on tomato could also be complex. The elongation of germ tubes is sensitive to humidity and relatively short germ tubes were generated at rh < 90% at all temperatures. However, no disease symptoms were observed on inoculated tomato plants when incubated in a greenhouse at 30 °C because rh was too low. This may explain the ecological distribution of this disease as it is present mainly in high humidity regions of the world where the humidity can be sufficient but the temperature is too low for most of the year. However, Yarwood et al. (1954) reported two optimal temperatures for *E. cichoracearum* according to their ecological distribution. They reported that the optimum temperature was 28 °C and 15 °C for the powdery mildew collected from a hot and cold region of California. However different criteria were used for the determination of these temperatures. The optimum temperature for the mildew collected from the hot region was based on lengths of hyphae *in vivo* and based on conidial germination on glass for that collected from the colder region.

Conidia of *E. cichoracearum* in this study were found to have a longer latent period (4-5 hr) than the 2 hr of *L. taurica* (Clerk and Ayesu-offei, 1967). Correll et al. (1988) reported that the onset of powdery mildew on tomato caused by *L. taurica* occurred at all stages of growth of the host whenever the inoculum was available. The disease developed better on tomato under dry conditions, but the opposite was found on pepper (Reuveni and Rotem, 1973). Caesar and Clerk (1985) reported that the low incidence of *L. taurica* on pepper during the dry season was possibly due to reduction in conidial germination and germ tube elongation caused by the water stress on plants during sporulation of the pathogen.

The primary infection stages of powdery mildew on tomato cv. Moneymaker were conidial germination, appressorium formation, and formation of secondary hyphae. These stages are in agreement with Masri and Ellingboe (1966) who

hyphae. These stages are in agreement with Masri and Ellingboe (1966) who reported similar stages on barley infected with *E. graminis* f. sp. *tritici*. Ten percent of conidia germinated on leaf discs of cv. Moneymaker 2 hr after inoculation and half of the germinated conidia had already formed appressorial initials. Secondary hyphae arose from 2.5% of conidia 18 h after inoculation. Masri and Ellingboe (1966) found that under optimum conditions for each stage, each phase was reached within a period of 4 hr. In this study haustoria were not seen even using different methods of clearing and staining.

The inhibition of conidial germination on the leaf surface of the wild genotypes of tomato LA458 and LA2747 and the reduction of germ tube lengths appear to be key contributory features of resistance of these lines to powdery mildew. These results concur with those of Staub et al. (1974) who reported a reduction in rate and extent of germination of *E. graminis* on cucumber leaves (non-host plant) which was about half of that on the host species barley. However, the germination of *E. cichoracearum* (pathogenic on cucumber) was normal on the leaves of barley (non-host), but the germ tubes were unable to penetrate the epidermal cells apparently due to inability to dissolve the epicuticular wax crystals (Staub et al., 1974). Cohen et al. (1987) found that the germination of *Peronospora tabacina* on the leaf surfaces of tomato, potato, pepper and tobacco was similarly high, but not on *Nicotiana debneyi* where the germ tubes were abnormal and appressorium formation was inhibited. Two mechanisms of resistance were recognized: preinfectional resistance in the form of reduced appressorium formation and postinfectional resistance associated with the collapse of epidermal plant cells (HR). In contrast, Mence and Hilderbandt (1964, 1965a, 1965b, 1966) reported that resistance of rose to powdery mildew *Sphaerotheca pannosa* was not related to conidial germination and prepenetration development of the fungus, but fewer haustoria developed in the epidermal cells of resistant

was associated with the death of haustoria-containing cells.

For the relatively few penetrations observed in resistant tomato genotypes cells responded in a hypersesitive-like manner and this was associated with yellow fluorescence. Toyoda et al. (1978) and Koga et al. (1980) reported yellow fluorescence on collapsed wheat cells invaded by *E. graminis* f. sp. *hordei*. Cohen and Eyal (1988) also reported a similar response by epidermal cells of resistant genotypes of *Cucumis melo* after 16-24 hr of inoculation with the powdery mildew *Sphaerotheca fuliginea*. In this study autofluorescing epidermal cells were always associated with the appressoria on the resistant cultivars, but never on the susceptible cultivar, which is in agreement with Cohen and Eyal (1988, 1990). The yellow-fluorescing substance(s) was probably a phenolic(s) produced or released by the epidermal cells upon fungal invasion. Similarly accumulation of phenolic compounds at infection sites was reported by Stewart and Mansfield (1985) on onion bulb scales inoculated with *Botrytis allii* 24 h after inoculation. Accumulation of lignin-like material and callose in association with resistance to powdery mildew and other pathogens were reported by many authors on wheat and barley e.g. wheat against *Puccinia graminis* f. sp. *tritici* (Tiburzy and Reisener, 1990), barley against *E. graminis* f. sp. *hordei* (Koga et al., 1988), wheat against *Puccinia recondita* f. sp. *tritici* (Southerton and Deverall, 1990).

The germination inhibitor(s) was presumably from the leaf surface of the resistant tomato genotypes as it was released simply by washing unwounded leaves; both water and chloroform washes contained inhibitory activity, but the effect was more pronounced with chloroform washes. The antifungal properties of substances associated with the leaf surfaces of plants have been frequently reported (Blakeman, 1975; Greshon and Shank, 1980; Yang and Ellingboe, 1972; Blakeman and Atkinson, 1976; Godfrey, 1976). Waxes are complex mixture of lipids covering the plant surface mainly to protect it from water loss and other environmental hazards (Kolattukudy, 1970). Antifungal properties of fatty acids

lipids covering the plant surface mainly to protect it from water loss and other environmental hazards (Kolattukudy, 1970). Antifungal properties of fatty acids (n-alkanols, α,ω-n-alkanediols and ω-chloro-α-alkanols) were suggested to be influenced by the chain length of carbon atoms (Gershon and Shanks, 1980). The maximum toxicity was found when the chain was made up of 10 carbon atoms. Martin et al. (1957) found that ether soluble fractions of apple wax reduced the germination of conidia of powdery mildew *Podosphaera leucotricha* and suppressed the formation of lesions of *Botrytis fabae* on broad bean. A water-soluble acidic fraction was also suggested to have a role in the defense of apple leaves against *Podosphaera leucotricha* (Martin et al., 1957). The wax of beetroot leaves, extracted by chloroform and reformed on glass fibre, was also found to inhibit the germination of *Botrytis cinerea* spores (Blakeman and Sztejnberg, 1973). There was some inhibition of spore germination on wax deposited for 6 h and a marked inhibition after 18 h or longer (Blakeman and Sztejnberg, 1973). This inhibition of spore germination was probably associated with chemical rather than physical properties. However, in this study the washes of both water and chloroform were desiccated for 7 days to dry on cover slips before their use for the germination tests, and they maintained the inhibitory effects. Dix (1974) reported that water extracts of leaves of *Acer platanoides* inhibited the germination of spores of *Cladosporium herbarum, C. sphaerospermum* and *Cylindrocarpon radicicola.* The inhibitory effect was seasonal and increased linearly as the concentration of leaf material increased logarithmically. Blakeman and Atkinson (1976) reported that diethyl ether extracts of the water soluble fraction of wax extracts from broad bean, beetroot, lettuce and tomato inhibited the germination of conidia of *Botrytis cinerea.*

The inhibitory effect of water and chloroform washes from resistant and to a lesser extent from the susceptible genotype of tomato used in this study suggest that these compounds are present naturally on the leaf surfaces, but in higher

concentration on the resistant tomato accessions LA2747 and LA458. Resistant sugar beet leaf washings were reported to contain more inhibitor for the germination of conidia of *Cercospora beticola* than the susceptible cultivars (Brillova, 1971 reviewed by Gordfrey, 1976). Future work should concentrate on the characterization of the inhibitory compounds from tomato which would also facilitate comparison between the two lines.

There were significant differences between the tomato somaclones screened for resistance to powdery mildew, but no level of resistance was evident that could be useful for breeding purposes. Possibly because the screening of these genotypes was performed in a controlled environment optimal for mildew development any partial resistance may not have been expressed.

Resistance to *E. cichoracearum* does not exist in the cultivated tomato. However, the high levels of resistance found in tomato lines LA2747 and LA458 is promising although the genetic background of this is not known, this information would be of value to predict both the ease of transfer in breeding programmes and likely the stability in the field. Obviously further study on these lines is warranted.

Chapter (6)

General Discussion

The results of this study suggest that the morphogenetic response of tomato cultivars is affected by the genotype, type of explant, length of culture period and the combination of plant growth substances. However, organogenesis of tomato callus in a two-stage culture system was difficult. Plants were successfully regenerated from explants of tomato cvs Moneymaker and Rootstock in a one-stage system. Some of these somaclones had tetraploid chromosome numbers, others showed phenotypic variation. Although only a small number of tomato somaclones were screened against *Erysiphe cichoracearum* and *Xanthomonas campestris* pv. *vesicatoria* in this study, some differences between the somaclones in their reaction to the two pathogens were observed. However, a level of resistance sufficient for breeding purposes was not found and more somaclones need to be screened under controlled environmental conditions and in the field. The potential of somaclonal variation for disease resistance has been reported for many plant species (Evans, 1989; Carlson, 1973; Toyoda et al., 1989a and 1989b; Krishnamurthi and Tlaskal, 1974; Hammerschlag, 1990). However the successful use of this technique depends on the chance of finding the required change which is presumably a random mutational event, so a large number of somaclones need to be screened.

The screening of tomato regenerants *in vitro* against *Xcv* strains would reduce the amount of work and the space needed for the evaluation of these somaclones dramatically. It would enable the pre-screening of large numbers of cell populations. This should greatly reduce the numbers of plants to be screened *in vitro*, if all somaclones are derived from resistant cell cultures. Hammerschlag (1990) used a detached-leaf bioassay to evaluate peach somaclones for resistance

to *Xanthomonas campestris* pv. *pruni*. This method of screening saved the original somaclones and it was possible to differentiate between resistant and susceptible genotypes. Another *in vitro* screening method used by Dunbar and Stephens (1989) which involved the inoculation of *Pelargonium* somaclones with *Xanthomonas campestris* pv. *pelargonii* distinguished resistant from susceptible somaclones.

Two features of the interaction between strains of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) with susceptible and resistant host plants (pepper and tomato) are worthy of note. The population of pathogenic *Xcv* strains increased in leaves of susceptible hosts (tomato or pepper) while the population of the non-pathogenic *Xcv* strain E1141 remained fairly stable in both susceptible and resistance leaves. These features were also found to be operating *in vitro* between susceptible tomato cells in suspension and pathogenic and non-pathogenic strains of *Xcv*. The pathogenic *Xcv* strains caused the highest cell leakage in leaves of the susceptible tomato cv. Rootstock and these strains also had the highest tomato cell killing ability *in vitro* at all growth stages of the suspension cultured cells (Figures 4.5 and 4.12-14). Bioassay of the co-culture medium for toxicity to tomato cells and effects *in vivo* should indicate whether the cell damage was caused by a toxic metabolite. However, the co-culture media in this study were not tested for their toxicity to tomato cells. Toxic metabolites have been used for the selection of resistant callus from which disease resistant plants have been regenerated (Hammerschlag, 1990).

Attempts to infect cells in culture with powdery mildew conidia have been reported to be difficult perhaps due to the biotrophic nature of these pathogens. Webb and Gay (1980) could not infect callus of rose, sycamore, wheat or pea with mildew conidia. Mence and Hilderbrandt (1966) failed to infect the callus of *Rosa virginiana* with *Sphaerotheca pannosa* conidia. Franzone et al. (1982) attempted to infect callus of barley with *E. graminis* f.sp. *hordei*, but this proved

to be another example of the difficulty of infecting undifferentiated cultures of plants with powdery mildew. They found that conidial germination was abundant both on infected leaf segments and on callus tissue, but colonies of powdery mildew only developed on the leaf segments. In contrast, *Erysiphe cichoracearum* has been cultured on sunflower (*Helianthus annuus*) tumour tissue with 2% success (Heim and Gries, 1953). Schnathorst (1959) also reported successful growth of this mildew on folded epidermal strips and mesophyll cells of lettuce (*Lactuca sativa*). However, *in vitro* techniques for infecting cell cultures with powdery mildew, and consequently selection of cell lines resistant to the fungus remain to be established.

The optimal environmental conditions for the germination of powdery mildew conidia and germ tube elongation may give a better understanding of the geographical distribution of the species of powdery mildew of tomato. However, the epidemiological implications appear to be difficult due to the complexity and difficulty of correlating responses under controlled experimental conditions and those in the field.

Correll (1986) found tomato accessions resistant to powdery mildew, however the screening of these genotypes was done in a commercial field without inoculation. The levels of resistance of *L. chilense* accessions LA2747 and LA458 were confirmed here as very high since these accessions were screened under controlled conditions optimum for disease development. Resistance was associated at least in part with the leaf surfaces of these lines and antifungal compounds were released readily by chloroform washes. Investigation of the nature and properties of the antifungal substance(s) using analytical procedures such as thin layer chromatography should help in the screening programmes for the resistance against the disease. Each component of the extract can be assayed for its inhibitory effect on conidial germination. This procedure was used by Blakeman and Atkinson (1976) for *Chrysanthemum* leaf extracts against *Botrytis cinerea*.

The use of resistance to powdery mildew in a tomato improvement programmes depends on the successful transfer of this character(s) to the popular cultivars of tomato. Different approaches can be adopted to transfer the powdery mildew resistance from *L. chilense* to the commercial cultivars of *Lycopersicon esculentum*. However, *L. chilense* is a strong outbreeder due to self-incompatibilty as a result of the exerted stigmas. In addition there are severe genetic barriers to intercrossing which separate *L. chilense* from the cultivated tomato. The pistils of these wild species will not accept pollen of *L. esculentum* and consequently flowers abscise (Taylor, 1986). Although fruit development was obtained in the reciprocal crosses, viable seeds were rare. In some cases the ovules of these reciprocal crosses may contain embryos that can be used in embryo culture. Rick and Lamm (1955) produced six hybrids of *L. esculentum* X *L. chilense* by embryo culture. This could perhaps be used to transfer the resistance against powdery mildew to *L. esculentum* cultivars. The use of *L. peruvianum* accessions, which are compatible with both *L. chilense* and *L. esculentum*, as a bridge to transfer this character is an alternative method to transfer this character.

Anther culture and somatic hybridization can be used for this purpose as reported by Zagorska and Palakarcheva (1976). They developed homozygous lines of tobacco with a complex resistance to blue mould (*Peronospora tabacina*) and to *E. cichoracearum* using anther and tissue cultures of 3 hybrids of tobacco. Haploid plants obtained in anther cultures were diploidized through cultures of leaves or petioles Zagorska and Palakarcheva (1976).

This programme was partially successful in developing a tissue culture technique for regenerating tomato somaclones. These somaclones showed genetic and morphological variation. The progenies of these somaclones showed differences in their interaction with *Xcv* strain E3. Cell suspension cultures were also used to investigate the interaction between tomato cells and *Xcv* strains and the possibility of toxin production in the coculture medium. Variation was found

between tomato cells at different phases of suspension culture growth in their interaction with *Xcv* strains. Actively dividing cells from the exponential phase showed more tolerance compared to those from stationary and lag phases. However, regeneration of tomato plants from these cells was not achieved on the combinations of plant growth substances used.

These results suggest that somaclonal variation is a potential method for the induction of new characters in plants and can be used as means of crop improvement. The interaction between powdery mildew and the tomato genotypes LA458 and LA2747 was made possible under the controlled conditions of *in vitro* techniques. The screening of these genotypes under environmental conditions favourable for powdery mildew development confirmed the initial resistance against powdery mildew claimed for these genotypes (Rick personal communication). To my knowledge, this is the first report of high levels of resistance to this pathogen on tomato genotypes which were screened under controlled conditions.

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Appendix 1:

Nutrient Yeast Glycerol Broth (NYGB)

Oxoid bacteriological peptone	: 5g
Oxoid yeast extract powder	: 3 g
Glycerol	: 20 g
Distilled water	: 1000 ml

(pH 7.0 before autoclaving)

NYGA

NYGB (prepared as described above)

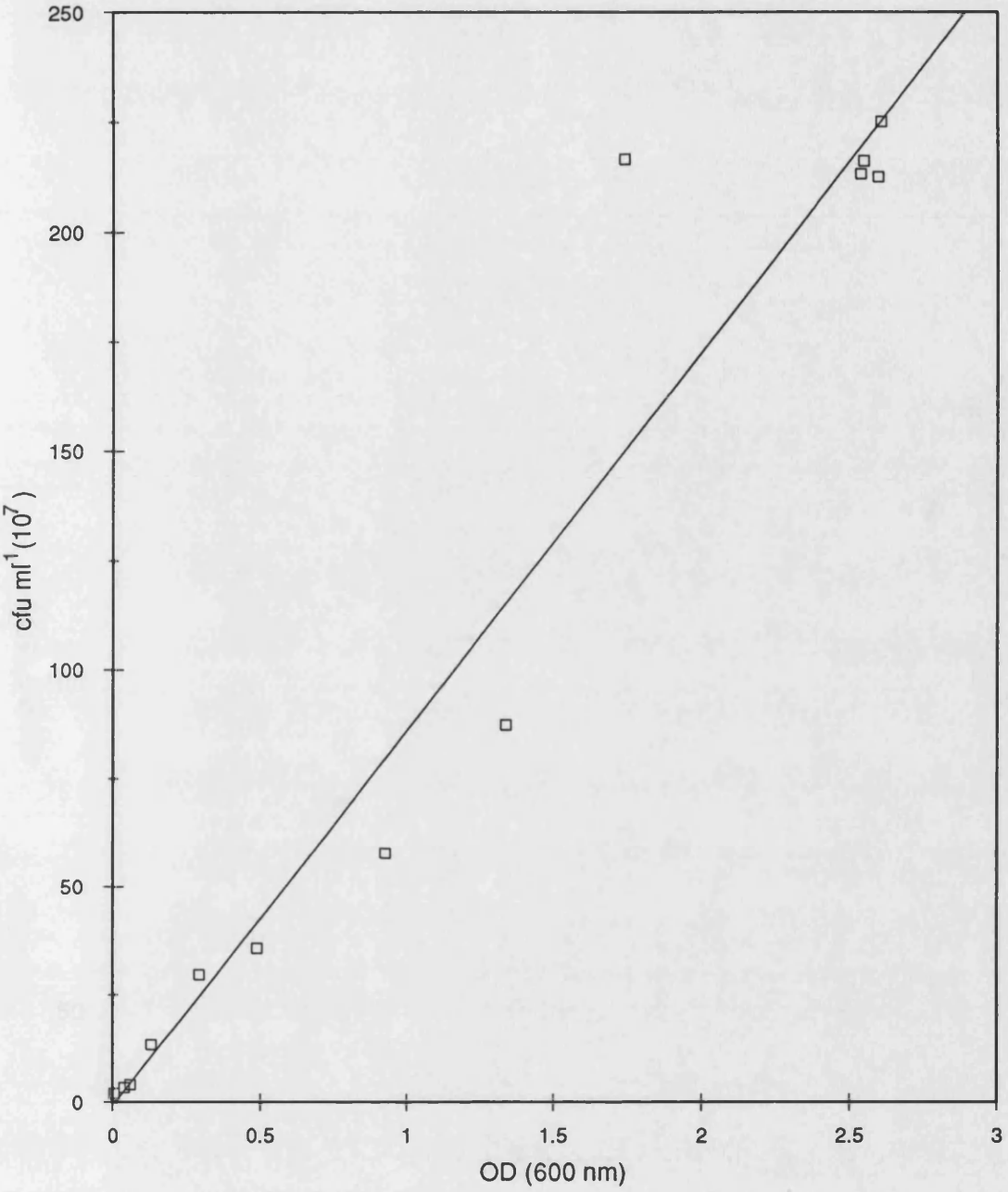
Oxoid tissue culture agar No. 1	: 14 g

Appendix 2:

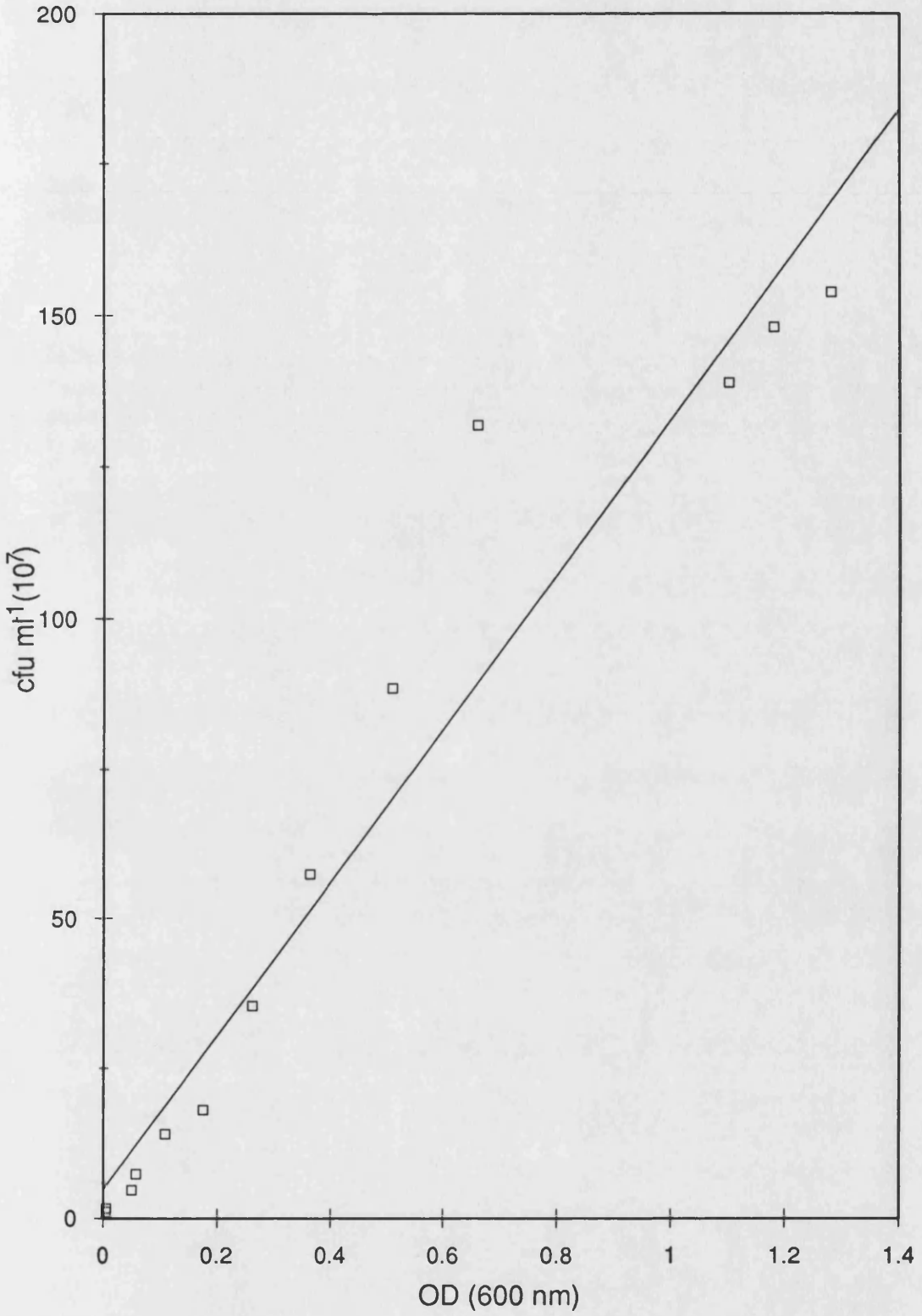
Modified Watanabe Broth

L-glutamic acid	: 1.0 g
Methionine	: 0.5 g
Sucrose	: 5.0 g
$(NH_4)_2HPO_4$	: 3.0 g
KH_2PO_4	: 2.0 g
$MgCl_26H_2O$	: 0.2 g
$FeSO_4.7H_2O$	: 0.01 g
$MnSO_4.7H_2O$	: 0.01 g
Distilled Water	: 1000 ml

(pH 7.0 before autoclaving)



Appendix 3: Growth of *Xanthomonas campestris* pv. *vesicatoria* strain E3 in nutrient yeast glycerol broth



Appendix 4: Growth of *Xanthomonas campestris* pv. *vesicatoria* strain E3 in modified Watanabe broth

Appendix 5:

Symptoms of Leaf Spot Disease on Pepper cv. ECW Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

ANOVA after squre root transformation (x + 0.5)

Source of Variation	DF	SS	MS	F	
Treatments	6	43.994	7.332	5.9	***
Between Plants	28	34.789	1.24246		
Plants	34	37.785			
Time	7	0.526	0.075	4.44	***
Time X Treatments	42	0.688	0.016	0.946	NS
Within Plants	183	3.0927	0.0169		
Total	279	83.089			

Appendix 6:

Symptoms of Leaf Spot Disease on tomato cv. Rootstock Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

ANOVA after squre root transformation (x + 0.5)

Source of Variation	DF	SS	MS	F	
Treatments	6	19.928	3.321	336.91	***
Between Plants	28	0.276	0.00986		
Plants	34	20.204			
Time	5	15.067	3.0134	753.35	***
Time X Treatments	30	16.20	0.54	135.0	***
Within Plants	140	0.557	.004		
Total	209	52.028			

*** Significant at ($p < 0.001$).

Appendix 7:

Conductivity of leaves Tomato cv. Rootstock infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

Source of Variation	DF	SS	MS	F
Treatments	6	3.68	0.61365	33.04 ***
Between Plants	28	0.52	0.0186	
Plants	34	4.2		
Time	5	7.5127	1.50255	2146.5 ***
Time X Treatments	30	1.78603	0.0593	85.04 ***
Within Plants	140	0.0933	0.0007	
Total	209	13.595		

Appendix 8:

Population (log transformation) of *Xcv* Strains in Tomato cv. Rootstock

Source of Variation	DF	SS	MS	F
Treatments	5	171.4	34.28	5193.93 ***
Between Plants	24	0.6	.0066	
Plants	29	171.56		
Time	5	71.93	14.39	1940.2 ***
Time X Treatments	25	64.27	2.57	346.5 ***
Within Plants	120	0.89	0.497	
Total	179	308.65		

*** Significant at ($p < 0.001$).

Appendix 9:

Symptoms of Leaf Spot Disease on tomato cv. Hawaii 7998 Infiltrated with *Xcv* Strains E3, E1141 and E1141(p6AD4)

ANOVA after squre root transformation (x + 0.5)

Source of Variation	DF	SS	MS	F	
Treatments	6	39.53	6.588	11.85	***
Between Plants	28	15.57	0.5561		
Plants	34	55.10			
Time	5	0.1731	0.0346	5.05	*
Time X Treatments	30	0.4718	0.0157	2.29	*
Within Plants	140	0.9592	0.00685		
Total	209	56.7043			

Appendix 10:

Conductivity of Tomato cv. Hawaii 7998 Leaves Inoculated by *Xcv* Strains (Angular Transformation)

Source of Variation	DF	SS	MS	F	
Treatments	6	6.21701	1.036	548.54	***
Between Plants	28	0.01529	0.00055		
Plants	34	6.2323			
Time	5	1.51402	0.3028	3028.0	***
Time X Treatments	30	3.55174	0.1184	1183.9	***
Within Plants	140	0.0164	0.0001		
Total	209	11.34064			

* Significant at ($p < 0.05$).

*** Significant at ($p < 0.001$).

Appendix 11:

Population (log transformation) of Xcv Strains in Tomato cv. Hawaii 7998

Source of Variation	DF	SS	MS	F
Treatments	5	138.54	27.7	511.38 ***
Between Plants	24	1.3	0.054	
Plants	29	139.84		
Time	5	32.37	6.4741	93.29 ***
Time X Treatments	25	23.318	0.9327	13.44 **
Within Plants	120	8.327	0.06939	
Total	179	203.855		

Appendix 12:

ANOVA of Survival of Tomato cv. Rootstock in Distilled Water and B5 Dilutions

Source of Variation	DF	SS	MS	F
Media	4	0.0017	0.0005	
D. Water vs. B5 Dilutions	1	0.0005	0.0004	0.29 NS
Within B5 Dilutions	3	0.0012	0.0017	0.23 NS
Residual	10	0.0172		
Flasks	14	0.189		
Time	6	1.6	0.26666	165.22
Time X Media	24	0.0624		***
Time X D. Water	6	0.026	0.0043	
Time X B5 Dilutions	18	0.0364	0.002	2.68 *
Residual	60	0.0968	0.0016	1.25 NS
Total	104	1.77832		

NS	Not significantly different.
*	Significant at ($p < 0.05$).
**	Significant at ($p < 0.01$).
***	Significant at ($p < 0.001$).

Appendix 13:

ANOVA of Tomato cv. Rootstock Cells (Lag Phase) Bioassay for Killing Ability of *Xcv* Strains (E3, E1141 and E1141(p6AD4))

(A) All Treatments:

Source of Variation	DF	SS	MS	F
Treatments	3	3550.00	1183.33	278.43 ***
Between Flasks	8	35.00	4.25	
Flasks	11	3584.00		
Time	6	10210.98	1701.83	634.91 ***
Time X Treatments	18	2628.17	146.01	54.47 ***
Within flasks	48	128.66	2.68	
Total	83	16551.81		

(B) Omitting the Control:

Source of Variation	DF	SS	MS	F
Xcv Strains	2	560.22	280.11	132.5 ***
Between Flasks	6	12.78	2.13	
Flasks	8	573.00		
Time	6	12379.30	2063.22	153.0 ***
Time X Strains	12	360.89	30.07	2.23 *
Within flasks	36	485.45	13.4847	
Total	62	3437.75		

(C) Omitting E1141:

Source of Variation	DF	SS	MS	F
Xcv Strains	1	2.88	2.88	2.796 NS
Between Flasks	4	4.12	1.03	
Flasks	5	7.00		
Time	6	10134.93	1689.16	457.715 ***
Time X Strains	6	31.62	5.27	1.42 NS
Within flasks	24	88.57	3.69	
Total	41	10262.12		

NS Not significantly different.

* Significant at ($p < 0.05$).

*** Significant at ($p < 0.001$).

Appendix 14:

ANOVA of Tomato cv. Rootstock Cells (Exponential Phase) Bioassay for Killing Ability of *Xcv* Strains (E3, E1141 and E1141(p6AD4))

(A) All Treatments:

Source of Variation	DF	SS	MS	F	
Treatments	3	3121.61	1040.54	371.79	***
Between Flasks	8	22.39	2.8		
Flasks	11	3144			
Time	7	11318.49	1616.93	386.53	***
Time X Treatments	21	3597.49	171.31	40.95	***
Within flasks	56	234.26	4.1832		
Total	95	18294.24			

(B) Omitting the Control:

Source of Variation	DF	SS	MS	F	
Xcv Strains	2	1057.58	528.79	155.3	***
Between Flasks	6	20.42	3.403		
Flasks	8	1078.00			
Time	7	12680.88	1811.55	366.50	***
Time X Strains	14	1884.42	134.6	27.23	***
Within flasks	42	207.58	4.942		
Total	71	15850.88			

(C) Omitting E1141:

Source of Variation	DF	SS	MS	F	
Xcv Strains	1	65.33	65.33	13.285	***
Between Flasks	4	19.67	4.9175		
Flasks	5	85.0			
Time	7	13363.00	1909.00	280.82	***
Time X Strains	7	72.33	10.33	1.52	NS
Within flasks	28	190.347	6.7978		
Total	47	13710.67			

NS Not significantly different.

*** Significant at ($p < 0.001$).

Appendix 15:

ANOVA of Tomato cv. Rootstock Cells (Stationary Phase) Bioassay for Killing Ability of *Xcv* Strains (E3, E1141 and E1141(p6AD4))

(A) All Treatments:

Source of Variation	DF	SS	MS	F	
Treatments	3	309.42	103.14	30.03	***
Between Flasks	8	27.48	3.435		
Flasks	11	336.9			
Time	3		344.75	135.22	***
Time X Treatments	9	1034.25	23.51	9.22	***
Within flasks	24	211.58	2.55		
Total	47	61.19			

(B) Omitting the Control:

Source of Variation	DF	SS	MS	F	
Xcv Strains	2	127.17	63.58	17.883	**
Between Flasks	6	21.33	3.555		
Flasks	8	148.5			
Time	3	1034.0	344.67	119.31	***
Time X Strains	6	127.5	21.25	7.35	***
Within flasks	18	52.0	2.8888		
Total	35	1362.0			

(C) Omitting E1141:

Source of Variation	DF	SS	MS	F	
Xcv Strains	1	42.67	42.67	12.999	*
Between Flasks	4	13.13	3.28		
Flasks	5	55.8			
Time	3	1012.8	337.61	154.63	***
Time X Strains	3	33.00	11.0	5.0382	**
Within flasks	12	26.2	2.183		
Total	23	1127.83			

* Significant at ($p < 0.05$).

** Significant at ($p < 0.01$).

*** Significant at ($p < 0.001$).

Appendix 16:

ANOVA of Survival of Tomato cv. Rootstock Cells (Exponential Phase) in Direct Contact with and Seperated from *Xcv* Strains E3

A: All treatments

Source of Variation	DF	SS	MS	F
Treatments	2	298.92	149.46	52.5 ***
Between Flasks	6	17.08	2.847	
Flasks	8	316.0		
Time	4	8762.48	2190.62	852.7 ***
Time X Treatments	8	1921.71	240.21	93.5 ***
Within flasks	24	61.66	2.569	
Total	44	11061.85		

B: Omitting E3 in direct contact

Source of Variation	DF	SS	MS	F
Treatments	1	37.97	37.97	50.125 ***
Between Flasks	4	3.03	0.7575	
Flasks	5	41.0		
Time	4	2986.12	746.53	218.028 ***
Time X Treatments	4	38.0	9.5	2.774 NS
Within flasks	16	54.79	3.424	
Total	29	3120		

NS Not significantly different at ($p < 0.05$)

*** Significant at ($p < 0.001$).